

**Study of the Regulation of MicroRNAs by Protein Phosphatase
1 During Memory Formation in Mammals**

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Dedicated to.....

Bari Ami, my grand mother, whose simplicity carried wisdom I could not find in philosophical texts

Abu, my father, whose honesty and grace will always live on as my guides

and, above all...

Ami, my mother, to whom I owe the first word I ever learnt, to the last I would ever teach

A handwritten signature in dark ink, appearing to read 'Ali Jawaid', written in a cursive, flowing style.

Ali Jawaid
Zurich, 2016

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Summary

Emerging evidence supports a role for microRNAs (miRNAs) in the regulation of synaptic plasticity and memory formation. We investigated if protein phosphatase 1 (PP1), a potent memory suppressor, can modulate the expression of miRNAs relevant for memory, and whether a dysregulation of PP1-dependent miRNAs is implicated in age-related cognitive decline (ARCD).

For this, we conducted deep-sequencing screenings for hippocampal miRNAs that are differentially regulated during memory formation, as well as, those from a transgenic mouse model of improved memory resulting from inhibition of nuclear PP1 (NIPP1*) in forebrain neurons. Specific miRNAs, notably miRNA cluster miR-183/96/182, were differentially expressed in the hippocampus of NIPP1* mice. The cluster showed a similar pattern of regulation in wild-type mice after training. Importantly, over-expression of the cluster in the adult mouse hippocampus enhanced long-term memory, whereas, its inhibition impaired memory. Mechanistic *in vitro* studies show that PP1 regulates the biogenesis of miR-183/96/182 in a transcription-independent manner by enhancing the activity of the microprocessor complex, which increases the level of miRNA precursors pre-mir-183, -96, and -182. This pathway likely involves receptor-activated SMAD proteins (R-SMADs) as intermediates.

To investigate a role for miR-183/96/182 and their regulation by nuclear PP1 in ARCD, their hippocampal expression was compared between young and aged mice. Aging increased nuclear PP1 activity in mouse hippocampus, which impaired the biogenesis of miR-183/96/182 at the post-transcriptional level. Over-expressing miR-183/96/182 in the hippocampus reversed the aging-related decline in memory. Furthermore, miR-183/96/182 were decreased in the brain samples from patients with amyotrophic lateral sclerosis (ALS), a neurodegenerative condition characterized by TDP-43 pathology and cognitive dysfunction in almost 50% of the patients. *In vitro* studies showed that TDP-43 regulates the microprocessor-mediated biogenesis of miR-183/96/182 in a PP1-dependent manner.

We provide novel evidence that nuclear PP1 regulates the biogenesis of specific miRNAs associated with memory. This regulatory pathway is implicated in ARCD and may contribute to cognitive dysfunction in certain neurodegenerative conditions.

Zusammenfassung

Neue Erkenntnisse deuten darauf hin, dass microRNAs (miRNAs) eine Rolle in der Regulierung synaptischer Plastizität und Gedächtnisbildung spielen. In dieser Studie haben wir untersucht, ob Proteinphosphatase 1 (PP1) - ein potenter Gedächtnishemmer - die Bildung von miRNAs im Zusammenhang mit der Bildung von neuen Erinnerungen modulieren kann. Des Weiteren wurde untersucht, ob eine Dysregulation von PP1-abhängigen miRNAs im Zusammenhang mit altersbedingtem kognitiven Verfall (age-related cognitive decline, ARCD) steht.

Um diesen Sachverhalt zu überprüfen haben wir „*deep-sequencing screenings*“ von hippocampalen miRNAs, welche während der Gedächtnisbildung unterschiedlich reguliert werden, durchgeführt. Zudem haben wir dieses screening von miRNAs in einem transgenen Mausmodell mit verbesserter Gedächtnisbildung resultierend aus der Hemmung von nuklearem PP1 (NIPP1*), in Neuronen des Vorderhirns untersucht. Spezifische miRNAs, vor allem der miRNA cluster miR-183/96/182, wurden im Hippocampus von NIPP1 Mäusen differentiell exprimiert. Dieser cluster zeigte ein ähnliches Regulierungsmuster in wild-typ Mäusen nach Training. Überexpression des clusters in adultem Hippocampus der Maus verbesserte das Langzeitgedächtnis, wobei seine Hemmung das Erinnerungsvermögen beeinträchtigt hat. Mechanistische *in vitro* Studien zeigen, dass PP1 die Biogenese von miR-183/96/182 in einer von der Transkription unabhängigen Art durch verbesserte Aktivität des *micropressor complexes* reguliert, welche die miRNA Vorläufer pre-mir-183, -96 und -182 erhöht vorkommen lässt. Dieser Signalweg beinhaltet Rezeptor-aktivierte SMAD Proteine (R-SMADs) als Reaktionsintermediate.

Um eine mögliche Rolle der miR-183/96/182 und deren Regulation durch nukleares PP1 in ARCD zu untersuchen, wurde deren hippocampale Expression zwischen jungen und gealterten Mäusen verglichen. Nukleare PP1-Aktivität war erhöht in gealterten Mäusen. Diese wiederum beeinträchtigte die Biogenese von miR-183/96/182 auf der post-transkriptionellen Ebene. Überexpression von miR-183/96/182 im Hippocampus wirkte dem

altersbedingten Gedächtnisverlust entgegen. Des Weiteren war miR-183/96/182 Gewebeproben von Patienten mit amyotroper lateraler Sklerose (ALS), eine neurodegenerative Krankheit charakterisiert durch TDP-43 Pathologie und kognitiver Dysfunktion in fast 50% der Patienten, reduziert. *In vitro* Studien konnten zeigen dass TDP-43 die „microprocessor-abhängige“ Biogenese von miR-183/96/182 in einer PP1-abhängigen Art reguliert.

Wir legen neue Erkenntnisse vor, dass nukleares PP1 die Biogenese von spezifischen miRNAs, welche im Zusammenhang mit Erinnerungen stehen, reguliert. Dieser regulatorische Signalweg steht im engen Zusammenhang mit ARCD und könnte zur kognitiven Dysfunktion in bestimmten neurodegenerativen Krankheitsbilder beitragen.

List of abbreviations (as they appear in the text)

STM: short-term memory

LTM: long-term memory

H.M: Henry Molaison

MTL: medial temporal lobe

PP1: protein phosphatase 1

miRNAs: microRNAs

LTP: long-term potentiation

LTD: long-term depression

CamKII: Calcium/Calmodulin dependent kinase II

PKC: protein kinase C

PKA: protein kinase A

CaN: Calcineurin

NMDA: N-methyl-D-aspartate

PSD95: post-synaptic density 95

CREB: cAMP response element binding protein

IEGs: immediate early genes

HDAC: histone deacetylase

RNA pol II: RNA polymerase II

RISC: RNA induced silencing complex

Zif: zinc finger protein

MeCP2: methyl CpG-binding protein 2

BDNF: brain derived neurotrophic factor

RAC1: Ras-related C3 botulinum toxin substrate 1

KCC2: Potassium chloride co-transporter 2

CPEB3: cytoplasmic polyadenylation element binding protein 3

MEF2D: myocyte-specific enhancer factor 2D

SYT1: synaptotagmin 1

NFkB: nuclear factor kappa-light-chain-enhancer of activated B cells

CPLX1: complexin1

NSF: N-ethylmaleimide sensitive fusion protein

A2BP1: ataxin 2-binding protein 1

Pri-mir/pri-mirna: primary microRNA

C-terminal: carboxy-terminal

N- terminal: amino-terminal

Pre-mir/pre-mirna: precursor microRNA

DGCR8: DiGeorge syndrome critical region 8

Receptor-activated SMAD: R-SMAD

TDP-43: TAR DNA binding protein of 43 kDa

HNRNP1: heterogeneous nucleus ribonucleoprotein A1 (HNRNPA1)

KSRP: KH type splicing regulatory protein

EXP5: exportin 5

RAN: RAs related nuclear protein

TRBP: TAR RNA binding protein

AGO: argonaute

eIF4G: eukaryotic translation initiation factor 4 G

lncRNAs: long non-coding RNAs

Xrn: exoribonuclease

ARCD: age-related cognitive decline

AD: Alzheimer disease

FTLD: frontotemporal lobar degeneration

PD: Parkinson disease

ALS: amyotrophic lateral sclerosis

CNS: central nervous system

FTD: frontotemporal dementia

NIPP1: nuclear inhibitor of PP1 (full length)

NIPP1*: nuclear inhibitor of PP1 (PP1 binding fragment)

N2a: mouse neuroblastoma cells

CD TDP: cognitive dysfunction associated with TDP-43 proteinopathies

LA: lateral amygdala

NOR: novel object recognition

NRG1: neuregulin-1

GRM5: glutamate metabotropic receptor 5

PP2A: protein phosphatase 2A

NUFIP2: nuclear fragile X mental retardation protein interacting protein 2

LNA: locked nucleic acid

PRKCZ: protein kinase C zeta

TGF- β : transforming growth factor beta

BMP: bone morphogenic protein

CLIP: cross-linked immunoprecipitation

ALK-5: activin receptor like kinase 5

PTSD: post-traumatic stress disorder

PP1R1A: PP1 regulatory sub-unit 1A

AKAP1: A kinase anchor protein 1

HU: hydroxyurea

DM2: diabetes mellitus type 2

Prologue: Understanding Memory

Memory formation is one of the most elegant functions of the brain that has been uniquely preserved among multicellular organisms, from simple creatures like *C. elegans* and *Aplysia*, to higher life forms like rodents and primates. The ability to register the information perceived by the sensory organs, storage of pertinent information in the context of its emotional associations, and its recall at a later stage is pivot of all executive functioning in humans. Memory formation is a precursor to almost all advanced human behaviors, ranging from language development to economic decision making. Indeed, who we are is defined by an intertwined collection of learning experiences. When the ability to learn is impaired in neurodevelopmental disorders, or when new memories cannot be formed and/or old ones are forgotten in neurodegenerative conditions or with aging, it leads to extreme morbidity in humans. Therefore, understanding how memories are formed in the brain, discovering which internal and external factors can impair this quintessential brain function, and unveiling any possibilities to preserve this astonishing feat are subjects of considerable neuroscience research.

Memory formation is a complex process, which employs diverse biological pathways based on its temporal persistence, the accompanying emotional responses, and the nature of the information stored. Based on its temporal persistence, memory can be classified as short-term memory (STM), lasting from seconds to hours, and long-term memory (LTM), persisting for days to years (Kandel et al. 1986). Based on its nature, memory can be *declarative*, a recallable collections of facts and events, or *procedural*, a learning of skills or habit development. From a neuropsychological perspective, memory formation can be divided into three inter-dependent processes: memory encoding/consolidation, which involves registration and storage of information; memory retrieval, which means recall of the memory trace; and memory reconsolidation, which refers to the subsequent modification of the originally stored memory trace (McKenzie & Eichenbaum, 2011).

Our understanding about the brain regions controlling declarative LTM formation benefited enormously from the study of Henry Gustav Molaison,

widely known as patient H.M. H.M. underwent medial temporal lobectomy as a treatment for temporal lobe epilepsy, resulting in bilateral resection of anterior two thirds of his hippocampi, para-hippocampi, entorhinal cortices, and the amygdalae. The surgery impaired H.M.'s ability to form long-term memories, with relative sparing of short-term and procedural memories (Scoville & Milner, 1957). Subsequent neuropsychological studies on H.M. established medial temporal lobe (MTL) as a critical memory system in the brain. To date, MTL, and in particular hippocampal formation, remain the most widely studied brain region in the context of learning and memory (Bird & Burgess, 2008). However, it must be mentioned that other brain regions, such as frontal cortex, may have important contributions to some forms of memory as well (Tronson & Taylor, 2007).

In this thesis, together with the help of my colleagues, I have attempted to further our knowledge about the molecular underpinnings of memory formation in mammals, and decipher pathways leading to its impairment with aging and in some disease conditions. I will introduce the elaborate mechanisms underlying LTM formation in the first chapter, and present our hypothesis about a potential role for protein phosphatase 1 (PP1) and micro RNAs (miRNAs) in their regulation. The second chapter will describe how certain miRNAs regulate memory in a PP1-dependent manner, a detailed investigation, which has now been accepted for publication in *Nature Communications*. The third chapter investigates a role for PP1 and miRNAs in memory impairment associated with aging and certain neurodegenerative conditions. This investigation has been written as a brief communication format. Finally, I have elaborated on the salient outcomes of my work in the 'Discussion' chapter, with an emphasis on the clinical implications of this thesis, and a plan for future investigations in the field.

1. Introduction

1.1. Molecular basis of memory

Memory formation is an intricate process involving tightly orchestrated sequence of molecular events, culminating into changes in neuronal communication (reviewed in Johansen et al. 2011, Kandel 2012). These changes reflect in the form of altered synaptic strength in an activity-dependent manner, known as synaptic plasticity (reviewed in Johansen et al. 2011). Depending on the frequency and timing of the stimulation, and the neuroanatomical regions it activates, neuronal signaling can either enhance synaptic transmission- long-term potentiation (LTP), or depress synaptic transmission – long-term depression (LTD). These electrophysiological changes are governed by unique signaling pathways, in which protein kinases and phosphatases occupy critical positions (Giese & Mizuno 2013, Baumgärtel & Mansuy 2012, Mansuy & Shenolikar 2006). Neuronal depolarization-induced entry of Calcium (Ca^{2+}) in neurons activates several kinases such as Ca^{2+} /Calmodulin dependent kinase II (CamKII), protein kinase C (PKC), protein kinase A (PKA), as well as, protein phosphatases protein phosphatase 1 (PP1), and Calcineurin (CaN). Together, these kinases and phosphatases regulate the expression and activity of important synaptic proteins, including synaptic receptors, channels, neurotransmitter, and neuromodulators (Kandel 2012, Baumgärtel & Mansuy 2012). For example, phosphorylation of different sub-units of N-methyl-D-aspartate (NMDA) receptors regulates their functionality (Sanz Clemente et al. 2012). Similarly, phosphorylation of different post-synaptic protein complexes such as post-synaptic density 95 (PSD95) and actin cytoskeleton control the response to synaptic activation (Nelson et al. 2013, Bertling et al. 2016). Further to these transient changes in protein expression and activity, *de novo* synthesis of proteins has been established as the hallmark signature of LTM (reviewed in Hernandez & Abel 2008).

Neuronal activity-induced formation of new proteins starts with translocation of the signals to the nucleus through second messenger molecules, where transcription factors critical for memory, such as cAMP- response element binding protein (CREB) are activated (reviewed in Johansen et al. 2011). The activation of these transcription factors directly or indirectly implicates neuronal

kinases CamKII, PKC, PKA, and phosphatases PP1 and CaN (reviewed in Johansen et al. 2011). These nuclear signaling events lead to transcription of multiple genes, including immediate early genes (IEGs), signaling molecules, as well as, components of the synaptic machinery (Loeblich & Nedivi, 2009). Importantly, many IEGs, such as c-Fos are transcription factors themselves, thus starting a cascade of *de novo* synthesis of cellular molecules, which is stringently regulated at each step (Ramirez-Amaya 2007).

Following transcription, the newly transcribed mRNAs are carried to the translational machinery components, in the neuronal soma and synapses for protein synthesis. Importantly, the process of mRNA transport, translation, as well as, the ensuing expression, activity, stability, and degradation of proteins are highly regulated in an activity-dependent manner (reviewed in Hernandez & Abel 2008).

1.2. Molecular basis of memory: the concept of memory inhibitors

It has been long argued that the brain needs an elaborate mechanism to prevent formation of useless or harmful memories (Silva & Josselyn, 2002). Indeed, amnesia for factual details of the event is extremely common in individuals exposed to trauma (reviewed in Brewin & Holmes, 2003, and Dalgleish, 2004). Similarly, it has been argued that forgetting in old age is attributed to impaired inhibition of memorizing useless details (Hedden et al. 2001).

The hypothesis that memory systems in the brain simultaneously employ memory-promoting and memory-suppressing pathways in a 'yin' and 'yang' like fashion to optimize formation of pertinent memories is well supported by electrophysiological and molecular studies. At the electrophysiological level, neuronal activity can induce the markedly contrasting LTP and LTD in the hippocampus, depending on the nature of the stimulation. Neuronal activity in the form of novel object exploration enhances LTP (Davis et al. 2004), whereas neuronal activity involving behavioral stress enhances LTD (Yang et al. 2005). Similarly, at the molecular level, a handful of memory suppressor molecules have been discovered in the last two decades. These memory suppressors constrain memory through a multitude of mechanisms; inhibiting the activity of

memory promoting transcription factors, interfering with relay of neuronal signaling underlying memory formation, or through blocking the expression of synaptic proteins (Table 1.1).

Table 1.1: Important molecular inhibitors of memory

Memory suppressor	Major memory suppressing mechanism(s)	Major Reference(s)
CREB-2	Inactivation of CREB-1	Bartsch et al. 1995, Abel et al. 1998, Chen et al. 2003
Calcineurin	Suppression of glutamate receptors and Zif268	Mansuy et al. 1998, Malleret et al. 2001, Baumgärtel et al. 2008
PP1	Epigenetic transcriptional inhibition of CREB and NFkB	Genoux et al. 2002, Kushibu et al. 2009,
miR-124	Post-transcriptional inhibition of CREB, Reduction in AMPA receptors	Rajasethupathy et al. 2009, Dutta et al. 2013 Gao et al. 2010 Siegert et al. 2015 Güven-Ozkan et al. 2016
miR-134	Post-transcriptional inhibition of BDNF	
miR-137	Impairment of pre-synaptic neurotransmitter release	
miR-980	Translational repression of autism susceptibility gene A2bp1	
HDACs	Transcriptional suppression of memory-promoting genes	Guan et al. 2009, Gräff et al. 2013
SLC22A transporter	Inhibitor of cholinergic signaling	Gai et al. 2016

1.3. Protein Phosphatases as epigenetic suppressors of memory

Protein phosphatases CaN and PP1 are key molecular regulators of memory that constrain learning. Both phosphatases are highly abundant in the brain, and when they are exclusively suppressed in the adult mouse forebrain by induction of specific inhibitors, memory formation is enhanced (Genoux et al. 2002, Malleret et al. 2001). On the other hand, increase in their activity in the brain experimentally or naturally during aging induces forgetting (Mansuy et al. 1998, Park et al. 2015). This is corroborated by experimental evidence showing that both CaN and PP1 regulate major forms of synaptic plasticity in the hippocampus and the amygdala (Jouvenceau et al. 2006, Winder et al. 1998, Malleret et al. 2001, Koshibu et al. 2011).

Off note, PP1 exerts its regulatory effect on memory through changes in the epigenome (Koshibu et al. 2009, 2011). Epigenetic changes, such as modifications of the histone code and chromatin remodeling accompany the effects of PP1 on memory at the behavioral level (Koshibu et al. 2009). These functions are mediated by the dephosphorylating action of PP1 on histone H3, as well as, modulation of other histone post-translational modifications (PTMs). Through these mechanisms, PP1 can act as a major transcriptional regulator during memory formation, regulating the RNA polymerase II (RNA pol II) occupancy at the promoter of CREB (Genoux et al. 2002).

Further to transcriptional regulation of memory-relevant gene expression, PP1 may modulate the expression of memory genes at the post-transcriptional level. Consistently, comparative analyzes of transcriptional and proteomic changes (measured by high throughput proteomic analyses) resulting from PP1 inhibition in the mouse hippocampus reveals a large dissociation between the two (unpublished data), strongly suggesting that post-transcriptional mechanisms of regulation operate in addition to transcriptional regulation to mediate the memory enhancing effect of PP1 inhibition.

1.4. Post-transcriptional regulation of gene expression in memory formation: Role of microRNAs

Formation and storage of LTM involve dynamic changes in the synaptic strength of neurons. Post-transcriptional gene regulation is particularly important for synaptic plasticity, as it provides a rapid and dynamic mode of control of synaptic proteins (Wang et al. 2012). This mode of control can be largely mediated by microRNAs (miRNAs/miRs), a class of small non-coding RNAs (20-22nt), which can regulate multiple targets through translational repression or degradation of their mRNAs (reviewed in Krol et al. 2010).

There is a strong biological rationale supporting a critical role for miRNAs in post-transcriptional regulation of memory-relevant gene expression. Many miRNAs are expressed in the brain, mostly in a region specific manner. Their biogenesis, functions, and turnover allow for a rapid and reversible regulation of gene expression (Wang et al. 2012). Typically, a single miRNA can target multiple mRNAs, allowing for a combinatorial regulation of gene expression (Krol et al. 2010), suitable for a phylogenetically superior brain function, such as memory formation.

Neuronal activity, the harbinger to memory formation, alters the expression of several miRNAs in brain regions governing cognitive functions, such as hippocampus, frontal cortex, and the amygdala. Importantly, the predicted targets of these miRNAs often contain a considerable proportion of synaptic proteins (Sim et al. 2014). Neuronal synapses are enriched for components of miRNA biogenesis machinery, as well as, the RNA induced silencing complex (RISC), where miRNAs can potentially regulate the synthesis of around 90% of the known synaptic proteins. (Paschou et al. 2012)

When components of miRNA biogenesis complex are experimentally suppressed or over-expressed, they perturb memory. Reduced synaptic expression of *Armitage*, a component of the RISC complex, impairs LTM formation in *Drosophila* (Ashraf et al. 2006). Similarly, conditional knockout of *Dicer* in the forebrain neurons enhances fear memory in mice (Konopka et al. 2010).

Furthermore, individual miRNAs are known to regulate different forms of memory (Saab & Mansuy, 2014, Table 2). The most widely characterized miRNAs in this regard are miR-212 and miR-132 (genomically expressed as a single cluster, miR-212/132), which are up regulated in the hippocampus during memory formation in mice (Wayman et al. 2008, Hansen et al. 2010). Further, hippocampal up regulation of miR-132 at levels comparable to physiological memory formation enhances learning in mice, whereas their excessive over-expression impairs memory (Wang et al. 2013). At the cellular and molecular level, miR-212/132 cluster regulates dendritogenesis, dendritic arborization, and LTP (Luiqart et al. 2010, Magill et al. 2010, Remenyl et al. 2013). Similarly, impairment of LTP and formation of long-term fear memory are observed in mice after hippocampal over-expression of miR-134 (Gao et al. 2010). Besides these, miR-34a, miR-128b, miR-182, miR- 137, miR-9, and miR-980 have also been shown to regulate the molecular mechanisms underlying LTP and/or memory formation (Table 2). Generally, these miRNAs contribute to memory formation by regulating the synthesis of cytoskeleton components, transcription factors, and trophic factors, which are responsible for dendritic remodeling, synaptogenesis, and synaptic plasticity (Saab & Mansuy, 2014, Woldemichael & Mansuy, 2016). Overall, the regulatory networks established by miRNAs in the brain allow them to play a critical role in memory formation and cognitive functions (Wang et al. 2012). However, although their contribution is well established, their mechanism of action, and particularly modes of regulation remain mostly unknown.

Table 1.2: Major memory-regulating miRNAs

miRNA	Key memory-relevant target(s)	Role in memory	Species	Major Reference(s)
miR-124	CREB, Zif268	First memory suppressor miRNA identified	Aplysia, mouse	Rajasethupathy et al. 2009, Yang et al. 2009
miR-128b	CREB1, Ppp1cc	Regulation of fear extinction memory	Mouse	Lin et al. 2011
miR-132/212	MeCP2	Bidirectional regulation of hippocampal memory	Mouse, rat	Hansen et al. 2010, Scott et al. 2012, Wang et al. 2013, Hernandez-Rapp et al. 2015, Hansen et al. 2016
miR-134	BDNF, Limk 1	Suppresses hippocampal memory	Mouse	Gao et al. 2010
miR-182	Cortactin, RAC1	Suppresses auditory fear memory	Mouse	Griggs et al. 2013
miR-34a	Notch signaling	Promotes fear memory consolidation	Mouse	Dias et al. 2014
miR-92	KCC2, CPEB3, MEF2D	Promotes contextual fear memory	Mouse	Vetere et al. 2014
miR-137	CPLX1, NSF, SYT1	Suppresses hippocampal memory	Mouse	Siegert et al. 2015
miR-9	Genes involved in cell-adhesion, endocytosis, and cell-death	Suppresses spatial-memory	Mouse	Malmevik et al. 2016
miR-980	A2BP1	Suppresses olfactory memory	Drosophila	Guven-Ozkan et al. 2016

1.5. Biogenesis of miRNAs

Most miRNAs are produced through the well-characterized canonical pathway of miRNA biogenesis in the cells, involving both transcriptional and post-transcriptional steps (reviewed in Ha & Kim, 2014).

Nuclear Biogenesis

In mammals, miRNAs are mostly encoded by genomic introns of both protein coding and non-coding genes. When miRNA sequences are present in the introns of protein-coding genes, they may share the transcriptional promoters with the host genes. However, a substantial number of miRNA promoters, distinct from the promoters of the host genes, have now been identified (Ozsolak et al. 2008, Monteys et al. 2010).

In some cases, individual miRNA sequences exist in close proximity to each other forming polycistronic transcription units (Lee et al. 2002). miRNAs belonging to the same cluster, are often functionally identical with over-lapping targets. Members of a cluster are usually co-transcribed, but can be subjected to distinct post-transcriptional regulations (Ha & Kim, 2014).

miRNA sequences are transcribed mostly by RNA pol II, and occasionally by RNA pol III (Cai et al. 2004, Lee et al. 2004, Pfeffer et al. 2005). The product of transcription is a double stranded (ds) long (>1 kb) RNA molecule called primary miRNA (pri-mir/pri-mirna). A typical pri-mir consists of a stem containing the mature miRNA sequence, a terminal loop, and single-stranded RNA segments at each end (Fig. 1.1).

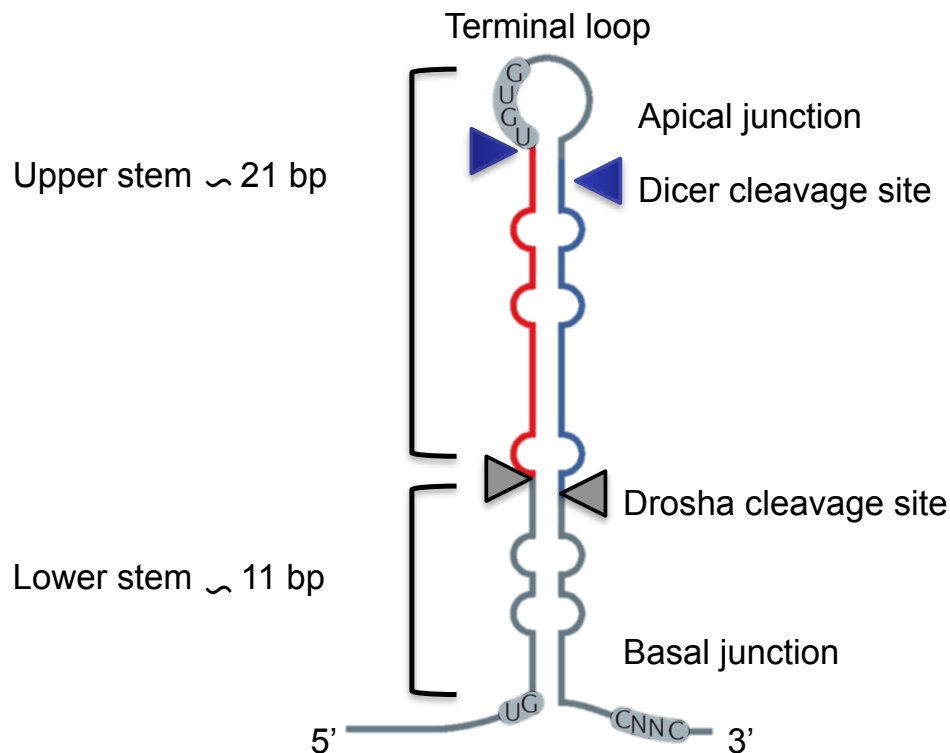


Fig.1.1. Structure of a primary microRNA containing the stem, terminal loop, and the single-stranded RNA segments at each end (adapted from Ha & Kim, 2014). Drosha along with DGCR8 cleaves the stem from the single-stranded RNA segments approximately 11 bp from the basal junction. The resulting pre-mirna undergoes further cleavage by Dicer, which removes the terminal loop to produce the mature miRNA guide and passenger strands.

The ds-stem of pri-mir is critical for its recognition and processing by Drosha, a nuclear RNAase III endonuclease vital for miRNA biogenesis (reviewed in Lee et al. 2002, Ha & Kim, 2014). The carboxy-terminal (C-terminal) of Drosha is essential for its primarily nuclear localization (Tang et al. 2010), whereas its RNA-binding and RNAse functions are related to its amino-terminal (N-terminal) (Gregory et al. 2004). Drosha requires the aid of another nuclear protein DiGeorge syndrome critical region 8 (DGCR8) for its binding to ds-RNA (Han et al. 2006). Together, Drosha and DGCR8 form a complex in the nucleus, known as the microprocessor complex, which is indispensable to miRNA biogenesis (Gregory et al. 2004, Han et al. 2006). The microprocessor cleaves the pri-mir hairpin at approximately 11 bp from the basal junction of the

stem to generate the ds- precursor miRNA (pre-mir) in a sequence-independent manner (Zeng & Cullen, 2005, Fig. 1).

As the functioning of the microprocessor is crucial for miRNA biogenesis, its expression, activity, and specificity are tightly regulated through complex mechanisms (reviewed in Ha & Kim, 2014, Fig. 2). These include a homeostatic cross-regulatory loop between Drosha and DGCR8, where DGCR8 stabilizes Drosha, whereas Drosha destabilizes DGCR8 mRNA levels to prevent its over-expression (Yeom et al. 2006, Han et al. 2006). Further to this auto-regulatory loop, Drosha and DGCR8 expression and nuclear localization depend on PTMs, such as phosphorylation (Tang et al. 2010, Tang et al. 2011, Herbert et al. 2013, Yang et al. 2015) and acetylation (Wada et al. 2012, Tang et al. 2013).

Similarly, the microprocessor processing can be selectively enhanced or diminished for certain miRNA by the action of some microprocessor 'accessory' proteins. These proteins are generally RNA binding proteins that selectively interact with Drosha and/or certain pri-mirs in a sequence-dependent manner (reviewed in Ha & Kim, 2014, Fig. 2). Notably, nuclear proteins p68 and p72 control the biogenesis of a subset of miRNAs by associating with the microprocessor (Fukuda et al. 2007). Some other proteins, such as receptor-activated SMAD proteins (R-SMADs) and p53 promote microprocessor activity through their interaction with p68 (Davis et al. 2008, Suzuki et al. 2009, Davis et al. 2010). Another DNA/RNA binding protein TAR DNA-binding protein of 43 kDa (TDP-43) interacts with Drosha to specifically promote the biogenesis of a few miRNAs (Kawahara et al. 2012, Di Carlo et al. 2013). Further to these, the microprocessor activity can be selectively modulated by heterogeneous nucleus ribonucleoprotein A1 (HNRNPA1), KH type splicing regulatory protein (KSRP), and LIN28 (Guil et al. 2007, Trabucchi et al. 2009, Qiao et al. 2012, Fig. 1.2)

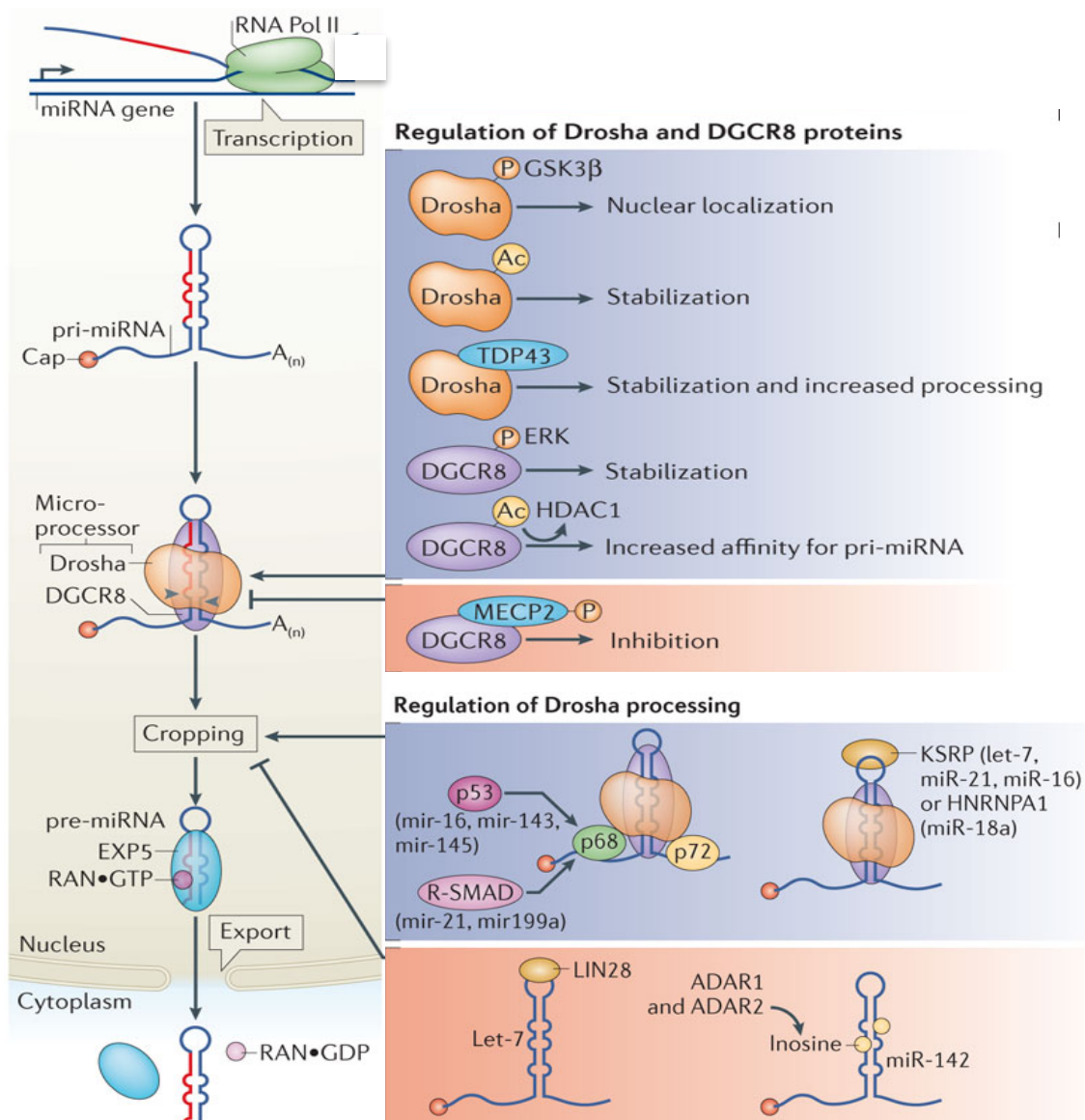


Fig.1.2. Schematic model of miRNA nuclear biogenesis and export into the cytoplasm with examples of how miRNA biogenesis can be regulated post-transcriptionally (reprinted from Ha & Kim, 2014).

Nuclear Export

Following Drosha processing, pre-miRNAs are exported into the cytoplasm through non-selective nuclear transporter exportin 5 (EXP5). EXP5 forms a transport complex with RAN-GTP and pre-mir, releasing the pre-mirna into the cytoplasm after GTP hydrolysis (Yi et al. 2003, Bohnsack et al. 2004, Lund et al. 2004). Knockdown of the gene encoding EXP5 results in reduction in miRNA levels, surprisingly without an accumulation of pre-mirs in the nucleus, suggesting that excessive pre-mirs in the nucleus are possibly degraded by endonucleases (Yi et al. 2003). Mechanisms governing the nuclear export of pre-mirs, and its disease-relevance remain poorly investigated as of now.

Cytoplasmic Biogenesis

In the cytoplasm, pre-mirs undergo cleavage at their terminal loops by another RNase III enzyme Dicer (reviewed in Ha & Kim, 2014). Similar to Drosha, N-terminal of Dicer is used for pre-mir identification and binding to their terminal loops (Gu et al. 2012), whereas its C-terminal carries the RNase function (Zhang et al. 2002, Zhang et al. 2004).

Conversion of pre-mir into mature miRNAs by Dicer is fundamentally a non-selective process. However, proteins that interact with Dicer to confer selectivity to its processing have been identified (reviewed in Ha & Kim 2014). Most notable, among these proteins is TAR RNA binding protein (TRBP), which promotes the Dicer processing for a miRNA subset, and also modulates their length (Fukunaga et al. 2012, Wilson et al. 2015, Lee et al. 2012). Mutations of *TARBP2*, the gene coding TRBP leads to altered miRNA profile in many cancer types (Melo et al. 2009). Further to TRBP, many other RNA binding proteins, like TDP-43, KSRP, and LIN28 also affect the activity of Dicer (Ha & Kim, 2014). Finally, Dicer expression seems to be homeostatically regulated by the level of mature miRNAs present in the cell, as there are binding sites of let-7 miRNA on Dicer mRNA, providing a negative feed-back loop for keeping Dicer expression and activity in check (Tokumaru et al. 2008, Jakymiw et al. 2010).

1.6. Functions of microRNAs

Upon completion of Dicer processing, dsRNAs of 20-24 nt length are generated. One of these two strands, the guide strand, is loaded into the miRISC complex, whereas the other strand, the passenger strand, is degraded by nucleases (reviewed in Ha & Kim, 2014). The strand selection for RISC loading depends on the thermodynamic properties of the strands, with a preferential loading of the strand with weaker 5' end pairing (Meister, 2013, Yates et al. 2013).

The miRISC complex comprises argonaute (AGO) protein and its partner GW182. Together these proteins interact with the miRNA and guide the complex to its target mRNAs (Meister, 2013). The binding between the

miRNAs and their targets is usually partially complementary, and depends on the miRNA seed sequences. mRNA targets usually, but not always, contain a target partially matching the miRNA seed sequence in their 3'UTR (Fabian et al. 2010, O' Carroll & Schaefer, 2013). The recruitment of targets mRNAs by the miRISC complex results in their translational repression or degradation. miRNAs inhibit the translation of their target mRNAs by interfering with the assembly of translational machinery. This includes impairment in the target mRNA association with eIF4G, ribosomal subunits, and translational elongation factors (Wilczynska & Bushell, 2015). Similarly, miRNAs can degrade their targets through recruitment of deadenylase complexes, which remove poly-A tails from the mRNAs, rendering them vulnerable to the exonuclease attack (Fabian et al. 2010, Fabian & Sonenberg, 2012).

The extent of target gene suppression by miRNAs is determined by several factors, including the baseline expression of miRNAs and their targets, as well as, presence of competitive endogenous RNA sponges, such as long non-coding RNAs (lncRNAs) (Wilczynska & Bushell, 2015, Thomson & Dinger, 2016). Single cell analysis of miRNA-mediated target suppression suggests that this suppression is determined by a minimal threshold expression of the target gene. Target expression below this threshold leads to a stronger suppression by miRNAs, thus enabling the miRNAs to act as a biological switch, which needs to be turned off to allow expression of the target. Contrary to this, when a target is expressed in abundance, the miRNA suppression is minimal and only used for fine-tuning (Mukherji et al. 2011). Further to that, many miRNAs function in a combinatorial fashion to suppress functionally identical mRNAs, and hence the level of suppression of a single target may not fully reflect the functional effect of miRNA manipulation (Balaga et al. 2012)

1.7. miRNA Degradation

The stability of mature miRNAs depends on their 5' and 3' thermodynamicity, as well as, the association between miRNAs and their mRNA targets (reviewed in Ha & Kim, 2014). Untemplated addition of adenosine or uracil residues at 3' miRNA ends renders them unstable (Heo et al. 2009, Jones et al. 2009). Similarly, when miRNAs are not bound to their targets, they are unloaded from the RISC complex through the action of exoribonucleases Xrn-1 and Xrn-2,

and rapidly degraded in the cytoplasm (Chatterjee & Grosshaus 2009, Finnegan & Pasquinelli 2013). Although, not much is known about the modes of miRNA degradation, emerging evidence suggests that a number of miRNAs exhibit dynamic turn-over (Krol et al. 2010, Gantier et al. 2011). This dynamic turn-over is an intrinsic property of a sizable fraction of brain miRNAs, which undergo simultaneous decrease and transcriptional increase in response to neuronal activity (Krol et al. 2010).

1.8. Regulation of microRNA biogenesis and functions: Potential role of PP1

While miRNAs are important regulators of memory processes, their biogenesis and activity can in turn, be potentially controlled by molecular mechanisms implicated in memory, such as protein phosphatases.

Protein phosphatases govern the activity of many transcription factors, some of which may regulate the transcription of miRNAs. For example, transcription factor CREB, which is potentially under the control of PP1 (Genoux et al. 2002, Koshibu et al. 2009), was shown to up-regulate miR-132 during activity-dependent synaptic remodeling (Nudelman et al. 2010). Similarly, nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB), a transcription factor inhibited by PP1 (Gu et al. 2014), increases the expression of miR-125b and miR-146a (Pogue et al. 2009, Hill et al. 2015).

Further, protein phosphatases can post-transcriptionally regulate miRNA biogenesis through altering the phosphorylation-dependent expression and activity of major components of the miRNA biogenesis machinery. These include Drosha, DGCR8, TRBP, and AGO proteins of the miRISC complex. Sub-cellular localization, stability and activity of Drosha and DGCR8 depend on phosphorylation/dephosphorylation of their C- and N-terminals (Tang et al. 2010, Tang et al. 2011, Herbert et al. 2013). Similarly, phosphorylation of TRBP generates miRNAs with greater stability (Paroo et al. 2009). Finally, phosphorylation of human AGO proteins controls the loading of miRNAs into the miRISC complex (Rüdel et al. 2011).

Given the dependence of the miRNA biogenesis machinery on phosphorylation, and the known link between protein phosphatases and memory formation, it is postulated that PP1 can impact the biogenesis and activity of miRNAs during memory formation. Further to that, it is critical to study the involvement of this regulatory pathway in conditions associated with memory impairment in humans.

1.9. Age Related Cognitive Decline (ARCD): Potential Involvement of PP1 and miRNAs

Aging is associated with a progressive impairment of learning abilities and memory formation, which can be a cause of considerable morbidity in the elderly. These deficits in memory vary in their manifestation and severity, ranging from occasional lapses in memory- 'senior moments', to widespread deficits in multiple cognitive domains (reviewed in Hedden et al. 2004, Konar et al. 2016). Longitudinal studies on brain functioning in the elderly reveal progressive deficits in especially episodic LTM (Hedden et al. 2001, Nillson et al. 2003, Hedden et al. 2004). These deficits occur without any clear indication of Alzheimer disease (Petersen et al. 2006, Jicha et al. 2006), and hence do not implicate neurodegeneration as the major underlying mechanism.

The exact molecular mechanisms underlying age related cognitive decline (ARCD) remain elusive. There are multiple reasons to believe that memory inhibitor PP1 could be implicated in ARCD. First, the activity of PP1 in hippocampal neurons increases with age in mice (Park et al. 2015). Second, transgenic inhibition of PP1 in forebrain neurons prevents age-related impairment of LTM in mice (Genoux et al. 2002). Third, strategies known to partially reverse or delay ARCD in mice, such as environment enrichment decrease PP1 activity in the brain (Park et al. 2015).

Similarly, a number of studies suggest a possible involvement of miRNAs in ARCD (Kosik et al. 2012). A deep sequencing comparison of whole brain miRNA expression between young and aged mice, showed a differential expression of 93 miRNAs with aging (Inukai et al. 2012). Recently, it has also been questioned if aging also impairs miRNA functioning by affecting their loading in the miRISC complex (Gregoriev & Bonini, 2014).

Based on the possible involvement of PP1 in miRNA regulation for memory formation, it becomes critical to ascertain if age-induced changes in activity of PP1 impair miRNA biogenesis and/or functioning to cause ARCD.

1.10. Cognitive Dysfunction associated with neurodegenerative disorders: Potential Involvement of PP1 and miRNAs

Increase in human life span during the last few centuries has resulted in a growing epidemic of neurodegenerative disorders, notably Alzheimer disease (AD), frontotemporal lobar degeneration (FTLD), Parkinson disease (PD), and amyotrophic lateral sclerosis (ALS). A common feature of these neurodegenerative disorders is abnormal intra-cellular and/or extra-cellular aggregation of proteins in the central nervous system (CNS) (reviewed in Ross & Poirier, 2004, Table 3). These deposits may be a result of genetic mutations in a fraction of familial cases, but the molecular mechanisms underlying these protein deposits in majority sporadic cases remains unclear. Down-stream to abnormal protein deposition, multiple pathways are activated, which are responsible for neuronal death and the clinical manifestations of these disorders. These include, notably, oxidative damage, glutamate excitotoxicity, and apoptosis (reviewed in Byrne et al. 2011).

Memory impairment, as a component of the constellation of neuropsychiatric symptoms collectively called dementia, is a cardinal feature of neurodegenerative disorders (Table 1.3). Both the human patients and animal models of neurodegenerative disorders have deficits in memory prior to any obvious indication of neuronal death (Rapp et al. 2002, Spires et al. 2006, Ford et al. 2015). Similarly, synaptic dysfunction precedes formation of senile plaques and neuronal death in models of AD (Wirths & Bayer, 2010). Further, brain amyloid beta deposits in these models interfere with the cascade of enzymes required for activation of memory promoter CREB (Vitolo et al. 2002, Arvanitis et al. 2007). Finally, a recent study revealed an epigenetic blockade on LTM formation in a mouse model of AD (Gräff et al. 2012).

Table 1.3: Pathological and Cognitive characteristics of common neurodegenerative disorders.

Neurodegenerative disorder	Pathological Neuronal aggregation	Severity and nature of cognitive impairment
Alzheimer disease (AD)	Senile plaques (amyloid β) Neurofibrillary tangles (Tau)	Early deficits in LTM progressing to dementia
Parkinson disease (PD)	Lewy bodies (α -synuclein)	Deficits in working memory in late stages
Lewy body dementia (LBD)	Lewy bodies (α -synuclein) Senile plaques (amyloid β)	Fluctuating deficits in STM, and/or working memory
Frontotemporal lobar degeneration (FTLD)	Tau (FTLD-Tau) TDP-43 (FTLD-TDP) FUS (FTLD-FUS)	Clinically sub-divided into behavioral variant FTD (deficits in working memory and executive functioning) or language variant FTD (deficits in language functioning or semantics). All variants show varying deficits in STM and LTM
Amyotrophic lateral sclerosis (ALS)	TDP-43	Progressive cognitive dysfunction similar to FTLD in 50% patients, severe enough to be classified as dementia in 20%

There are multiple reasons to query if cognitive dysfunction in neurodegenerative disorders implicates PP1 and miRNAs. An alteration of phosphatases and kinases may underlie the pathogenesis of multiple neurodegenerative disorders, as the pathological protein aggregates in these are often phosphorylated. This is the case, notably, for TDP-43 aggregates in ALS and FTLD (Neumann et al. 2006), fused in sarcoma (FUS) aggregates in FTLD (Neumann et al. 2009), tau aggregates in FTLD and other taupathies (Stanford et al. 2004), and alpha-synuclein (α -synuclein) aggregates in PD (Wang et al. 2012). Importantly, some of these proteins, for example TDP-43

and FUS also regulate the expression or activity of some phosphatases (Perera et al. 2014, Schwartz et al. 2012).

Similarly, TDP-43 and FUS regulate the activity of Drosha and Dicer (Kawahara et al. 2012, Di Carlo et al. 2013), thus making impaired miRNA biogenesis a candidate mechanism underlying cognitive dysfunction in neurodegenerative disorders. Indeed, TDP-43 and FUS proteinopathies have distinct miRNA expression profiles in the brain (Freischmidt et al. 2013, Modigliani et al. 2014).

Based on the aforementioned regulation of protein phosphatases and miRNAs by some proteins implicated in neurodegenerative disorders, it becomes important to study their role as mediators of cognitive dysfunction in the neurodegenerating brain.

1.11. Experimental aims

The main objectives of this thesis were to identify the major miRNAs that are regulated during LTM formation, and determine whether they are regulated by PP1-dependent pathways. Our particular focus was on the nuclear effect of PP1, since the early biogenesis of miRNAs occurs in the nucleus (Ha & Kim, 2014), and many neurodegenerative disorders hypothetically result from the loss of nuclear function of the pathologically aggregated proteins (Winklhofer et al. 2008). For this purpose, we initially took advantage of a transgenic mouse model of nuclear PP1 inhibition established in the Mansuy lab in which PP1 is inhibited selectively in the nucleus of forebrain neurons by expression of NIPP1*. In these mice, PP1 inhibition improves several forms of LTM (Koshibu et al. 2009). Further, we studied the activity of nuclear PP1 and its effect on miRNA biogenesis in aged (20-21 months old) wild-type mice, which show impairment in LTM (Pavlopoulos et al. 2013). The *in vivo* work in these mice was complemented by mechanistic studies on mouse neuroblastoma cells (N2a). We postulate that PP1 participates in the post-transcriptional control of gene expression in memory formation by regulating the level of memory-relevant miRNAs, and this pathway is likely implicated in ARCD and cognitive dysfunction in some neurodegenerative disorders. To investigate this hypothesis, we set the following experimental aims.

- 1) Identifying the miRNAs that are relevant for memory formation and are regulated by nuclear PP1
- 2) Identifying the targets of the miRNAs regulated by nuclear PP1
- 3) Functionally validating the role of these miRNAs in memory formation
- 4) Ascertaining the pathways by which PP1 regulates miRNA expression
- 5) Investigating if these miRNAs are associated with age-related memory loss and early cognitive dysfunction associated with certain neurodegenerative disorders

The first four experimental aims are addressed in Chapter 2 of this thesis, whereas, the fourth experimental aim is further elaborated on, and experimental aim 5 is exclusively covered in the Chapter 3.

2. The microRNA cluster miR-183/96/182 contributes to long-term memory in a protein phosphatase 1-dependent manner

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2.1. Abstract

Memory formation is a complex cognitive function regulated by coordinated synaptic and nuclear processes in neuronal cells. In mammals, it is controlled by multiple molecular activators and suppressors, one of which is protein phosphatase 1 (PP1), a signaling and transcriptional regulator that can also act as an epigenetic modulator. Here, we show that memory control by PP1 involves the microRNA cluster miR-183/96/182, and that this cluster is selectively regulated during memory formation. Inhibiting nuclear PP1 in neurons in adult mice *in vivo* or training wild-type animals on an object recognition task similarly increases the level of miR-183/96/182 in the hippocampus. Mimicking this increase by overexpressing miR-183/96/182 in the hippocampus enhances object memory, while suppressing endogenous level of the cluster reduces it. This effect involves the modulation of plasticity-related genes, signaling molecules and enzymes, and HDAC9 is identified as one of the functional targets. Further, PP1 controls these miRNAs in a transcription-independent manner involving the microprocessor-mediated generation of their precursors. These findings provide novel evidence for a key role for miRNAs in memory formation and newly suggest the implication of PP1 in miRNAs processing in the adult brain.

Main text**2.2. Introduction**

The formation of long-term memory depends on synaptic plasticity and on activity-dependent structural and functional changes in neuronal circuits. It is sustained by cascades of tightly orchestrated signaling molecules that positively or negatively regulate synaptic efficacy for the control of memory formation^{1,2}. One of the ultimate functions of these signaling cascades is the regulation of gene expression and the synthesis of new proteins necessary for the formation and the storage of long-term memory^{3,4}. In these cascades, protein kinases such as Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) and the cAMP-dependent protein kinase A (PKA) have a permissive role while protein phosphatases such as the Ca^{2+} /calmodulin-dependent protein phosphatase calcineurin (CN) and protein phosphatase 1 (PP1) act as memory suppressors^{5,6}.

PP1 is a ubiquitous phosphatase in the brain well positioned to orchestrate molecular processes resulting from neuronal activity, and is implicated in many brain functions. Our previous work has demonstrated that PP1 can regulate the expression of genes important for memory formation by influencing the epigenetic state of these genes, in particular, through posttranslational modifications of histone proteins⁷⁻¹⁰. PP1 is also an important regulator of gene transcription and RNA processing¹¹⁻¹³.

In recent years, microRNAs (miRNAs) have emerged as important regulators of gene expression in many biological systems. Most miRNAs identified so far are expressed in the brain and have distinct expression patterns at different developmental stages, and in different brain regions and cell types¹⁴⁻¹⁶. In addition, many miRNAs, along with components of the miRNA biogenesis and silencing machinery are enriched at synaptic sites¹⁷. So far several miRNAs have been implicated in neuronal functions including learning and memory formation^{18,19}. Yet, their mode of regulation in the context of cognitive functions remains poorly understood.

Here we show that a cluster of miRNAs comprising miR-183/96/182 is differentially regulated upon learning and is modulated by PP1. We provide

evidence that the production of miR-183/96/182 precursor is favored by the inhibition of nuclear PP1 in a transcription-independent manner, and that overexpression of the cluster in the hippocampus enhances memory in adult mice while its knock-down impairs memory. These effects are proposed to be mediated by regulation of miR-183/96/182 biogenesis and suppression of target genes such as HDAC9.

2.3. Results

2.3.1. MiR-183/96/182 expression is increased by learning or PP1 inhibition

To examine the role of PP1 in the regulation of miRNAs involved in memory formation, we took advantage of a transgenic mouse line in which the activity of PP1 can be inhibited inducibly in adult forebrain neurons by expression of a nuclear inhibitor of PP1, NIPP1 (NIPP1*)⁹. Previous work established that in these mice, inhibition of nuclear PP1 improves hippocampus-dependent forms of memory and causes widespread epigenetic and transcriptional changes of several genes⁸⁻¹⁰. Based on these findings and considering that PP1 can act as a transcriptional and post-transcriptional gene regulator, we postulated that inhibition of nuclear PP1 may alter the expression of miRNAs important for memory formation.

To test this hypothesis, NIPP1* animals and control littermates were trained on a novel object recognition (NOR) task, a paradigm based on the natural attraction of rodents for novel items that can elicit the formation of long-term memory (**Supplementary Fig. 1**). Following training, miRNAs were examined in the hippocampus by next generation deep sequencing. In total, over 84 million reads were sequenced, a large proportion of which (83%) corresponded to a size of 19-26nt with over 92% mapping to known mouse miRNAs (**Supplementary Fig. 2a, b**). The expression level of the identified miRNAs varied greatly, with some miRNAs being highly abundant, and others moderately or weakly abundant (**Supplementary Fig. 2c**). Overall, the level of most miRNAs was consistent across samples in NIPP1* transgenic and control littermates whether trained or not (**Supplementary Fig. 2d**), suggesting no gross alteration of miRNAs expression by PP1 inhibition or NOR training. Consistently, there was no global change in the expression of major

components of the miRNA biogenesis machinery by PP1 inhibition (**Supplementary Fig. 3**).

Differential expression analyses revealed that distinct sets of miRNAs are however up- or downregulated in NIPP1* mice compared to control littermates, and in NOR-trained mice compared to non-trained animals. Notably, a subset of miRNAs was similarly altered by PP1 inhibition and training (**Fig. 1a, b, Supplementary Fig. 4 and 5**). A closer look at these miRNAs revealed that the miR-183/96/182 cluster is upregulated in the hippocampus in both, NIPP1* transgenic mice and NOR-trained controls. Quantitative PCR (qPCR) confirmed a consistent increase (about 50%) in miR-183 and miR-182, while miR-96 was expressed at low level (**Fig. 1c, d**). These miRNAs have been implicated in neuronal activity and plasticity as well as in amygdala-dependent fear memory²⁰⁻²², and their predicted targets are involved in plasticity and neuronal signaling pathways (**Supplementary Fig. 15**, WebGestalt analysis tool).

To confirm the link between PP1 inhibition and upregulation of miR-183/96/182 cluster, PP1 was knocked-down in N2A cells using a pool of siRNAs targeting the 3'UTR of PP1 γ , an isoform predominantly linked to nuclear functions (**Supplementary Fig. 6a**). PP1 γ knockdown increased the level of miR-183 and miR-182 (**Supplementary Fig. 6b**), confirming that nuclear PP1 is implicated in the synthesis of these miRNAs.

2.3.2. PP1 inhibition affects the biogenesis of miR-183/96/182 precursors

We next examined the potential link between miR-183/96/182 cluster and PP1. MiRNAs are produced through a succession of biogenesis steps involving the transcription of primary miRNAs (pri-miRs), and their processing into precursor miRNAs (pre-miRs) in the nucleus then to mature miRNAs in the cytoplasm²³. To determine whether miR-183/96/182 biogenesis is modulated by PP1, we measured the level of pre-miR-183/96/182 in NIPP1* animals. In the hippocampus, pre-miR-183 and pre-miR-182 were up-regulated in the nuclear fraction and down-regulated in the cytoplasmic fraction (**Fig. 2a, b**). Similarly, neuronal activity induced by KCl treatment led to a rapid upregulation of pri-miR-183/96/182 and corresponding pre-miR transcripts in N2A cells

(Supplementary Fig. 6c). Combining PP1 γ knockdown and KCl stimulation caused a further increase in nuclear pre-miRNAs, which was reversed by overexpression of a PP1 γ construct carrying a siRNA-resistant open reading frame (**Fig. 2c**). Further, PP1 γ knockdown reduced the level of KCl-induced pri-miR-183/96/182 and cytoplasmic pre-miR-183/96/182 but had no effect on a control miRNA (**Fig. 2d, Supplementary Fig. 6d, e**), suggesting the selectivity of the effect. We next examined whether this action of PP1 requires gene transcription using the transcription inhibitor actinomycin D (ActD) (**Supplementary Fig. 7**). While ActD treatment significantly reduced pri-miRNA transcript (**Fig. 2d**), it had minimal effect on the up-regulation of pre-miRs induced by PP1 γ knockdown (**Fig. 2e**), suggesting that PP1 γ inhibition likely acts downstream of RNA Pol II-dependent transcription to regulate miR-183/96/182 level.

To further explore the mechanisms with which PP1 inhibition influences the level of pre-miR forms of the cluster, we conducted pri-miRNA processing assay. In this system, cropping of the stem-loop hairpin sequence from artificially introduced pri-miRNA transcript correlates with reduced firefly luciferase signal, indicative of pri-miRNA processing²⁴ (**Fig. 3a**). We observed that processing of both pri-miR-183 and pri-miR-182 was significantly increased upon inhibition of nuclear PP1 by NIPP1 overexpression (**Fig. 3b, c**), further strengthening our prior observation. Taken together, these results suggest that inhibition of PP1 γ facilitates the increase in miR-183/96/182 level triggered by neuronal activity by favoring pre-miRNA production at the microprocessor level.

2.3.3. PP1 inhibition replenishes the existing pool of miRNAs

Most neuronal miRNAs have a fairly rapid turnover following cellular activity, where changes in precursor miRNA transcription and processing precede changes in the level of mature miRNAs²¹. To examine whether this process is affected by PP1, we measured the level of miR-183/96/182 transcripts at different time points, with and without transcriptional inhibition. We observed that KCl stimulation decreases the level of mature miR-183/96/182 after 30 min but significantly increases it after 4hr (**Fig. 4a, b**). The early decrease was not affected by ActD treatment, but the increase at 4hr was reversed and even

inversed (leading to a decrease) by ActD (**Fig. 4c, d**), suggesting an initial usage and depletion of miRNAs induced by activity, followed by replenishment through increased biogenesis. Blockade of this replenishment by ActD results in continued miRNAs depletion. This blockade could be partially rescued by PP1 γ inhibition (**Fig. 4e**), confirming an effect of PP1 downstream of gene transcription.

2.3.4. Modulation of miR-183/96/182 in the mouse hippocampus affects long-term memory

Since the miR-183/96/182 cluster is up-regulated in the adult hippocampus following NOR training, we next examined whether inducing its expression at the time of learning has an effect on memory formation. We overexpressed miR-183/96/182 in hippocampus area CA1 in adult mice *in vivo* using a self-complementary adeno-associated virus (scAAV) vector expressing pre-miR-183/96/182 fused with GFP. Virus transduction and miRNA overexpression were confirmed by immunohistochemistry and qPCR (**Supplementary Fig. 9**). For NOR training (acquisition), the animals were exposed to three unfamiliar objects for 5 sessions of 5 minutes spaced by 5 minutes intervals, a protocol that induces robust long-term memory ⁷ (**Supplementary Fig. 10a**). MiR-183/96/182-overexpressing mice and controls explored the objects similarly during acquisition (**Supplementary Fig. 10b**). Likewise, both groups had comparable long-term object memory when tested 24hr after training (**Supplementary Fig. 10c, d**). Overall locomotor activity was similar in mice overexpressing miR-183/96/182 and controls (**Supplementary Fig. 10e, f**).

Previous studies on NOR and other memory paradigms have demonstrated that the duration and spacing of training sessions determine memory strength. For most paradigms, repeated and spaced training results in stronger memory than massed training ^{7,25-27}. Because our training protocol was intense, it may have masked the effect of miR-183/96/182 overexpression. Thus, we repeated this and all subsequent NOR experiments using a weaker protocol based on a single 10-min training session, followed by two test sessions 24 hours apart (**Fig. 5a**). Overall locomotor activity of the animals during open field test or object exploration during training was not affected by miRNA overexpression (**Supplementary Fig. 11a-c**). During test 1, both groups had a comparable low

level of memory, which increased during test 2 (**Supplementary Fig. 11d, e**), consistent with the notion that retrieval helps update and strengthen memory^{28,29}. Importantly, during the second test, miR-183/96/182 overexpressing mice had significantly better memory than control mice (**Fig. 5b**). To confirm the implication of miR-183/96/182 in long-term memory, we also expressed a sponge construct that competitively inhibits the miRNA cluster in the mouse hippocampus (**Supplementary Fig. 12a, b**). While sponge expression did not affect object exploration, it significantly impaired long-term memory 24hr after training (**Fig. 5c and Supplementary Fig. 12 d, e**). Taken together, these results provide evidence for the permissive role of the miR-183/96/182 cluster in the hippocampus in long-term object memory.

2.3.5. MiR-183/96/182 cluster regulates genes involved in neuronal functions and regulation including HDAC9.

Many of the predicted targets of the miR-183/96/182 cluster are involved in biological pathways relevant for neuronal signaling and plasticity, and epigenetic regulation (**Supplementary Fig. 15**). To validate some of these targets, we measured their level of expression in the hippocampus of mice overexpressing miR-183/96/182. Several genes including ion channels, receptors, a kinase, a phosphatase, and a histone deacetylase were significantly downregulated (**Fig. 6a**). We focused on one of these genes, HDAC9, which codes for a member of class II HDACs known to be an epigenetic regulator modulated by neuronal activity, and involved in the control of plasticity-related genes (Lang et al 2012, Lucio-Eterovic et al 2008, Mejat et al 2005, Sugo et al 2010). Histone acetylation and the enzymes that modulate acetylation such as HDACs, play a crucial role in the formation and storage of long-term memory³⁰ and HDAC inhibitors are increasingly appreciated as potential treatment options for cognitive deficits³¹. We therefore explored the link between the miR-183/96/182 cluster and HDAC9. We examined if HDAC9 is a direct target of miR-183/96/182 using a luciferase-based expression system containing a predicted miR-182 binding site of HDAC9 3'UTR. Upon miR-182 transfection, we observed destabilization of the construct, indicating targeting of the predicted HDAC9 target site by miR-182 (**Fig. 6b**). Further, the level of HDAC9 was reduced in the hippocampus of mice subjected to NOR training and testing compared to controls (**Fig. 6c**). To further evaluate the

importance of HDAC9 targeting by miR-183/96/182, we interfered with miR-182/HDAC9 interaction *in vivo* by injecting LNA modified target site blockers (TSB) (**Supplementary Fig. 14**). Interfering with miR-182 targeting of HDAC9 in mice overexpressing miR-183/96/182 cluster significantly reduced object exploration behavior during training and testing, without affecting novel object discrimination (**Fig. 7 c-e**). Together, these results identify HDAC9 as one of the mediating factors of miR-182/96/183 on cognitive processes.

2.4. Discussion

Our results newly reveal a novel role for the memory suppressor PP1 in the biogenesis of miRNAs during memory formation. They show that PP1 inhibition increases the level of the miR-183/96/182 cluster, and identify this cluster as an important modulator of memory formation. PP1 inhibition acts by enhancing the production of pre-miRNAs in the nucleus. This occurs in a background of mature miR-183/96/182 consumption upon neuronal stimulation and continued replenishment by increased transcription. These findings suggest that PP1 inhibition facilitates nuclear miRNA processing during neuronal activity, possibly by influencing the microprocessor complex.

Several studies have reported the involvement of protein phosphorylation in pri-miRNA processing, mainly through protein kinase-mediated regulation of the stability, interaction, and nuclear localization of components of the microprocessor complex^{32,33 34,35}. This study significantly extends these findings by newly showing that PP1 is involved in this processing. The precise modes of action of PP1 remain unknown but PP1 may modulate the processing of specific miRNAs by interacting with RNA binding proteins that contain a PP1 recognition motif³⁶. It could also act on splicing since it can interact with components of the spliceosome machinery, which is known to crosstalk with nuclear miRNA processing³⁷⁻⁴⁰. Binding of proteins such as Tra2-beta1, SF2A, Srp30c, and ASF to PP1 through conserved RNA recognizing domains is essential for correct splice site selection^{36,41} and may thus by extension, also affect miRNA processing. Interestingly, a recent study demonstrated a developmentally-timed processing of pri-miR-183/96/182 that is crucial for neuronal organization. This is mediated through its interaction with a component of the microprocessor complex – Ddx3x⁴². Our results further

corroborate the highly regulated processing that this cluster undergoes in neurons in response to external signals.

The present results further show that overexpression of miR-183/96/182 in the mouse hippocampus improves long-term object memory. Interestingly, this effect is observed following training with a weak (single session), but not a strong (spaced sessions) training protocol. This could be attributed to a 'spacing effect' where sessions separated over a period of time lead to stronger activation of molecular pathways needed for memory formation, including plasticity-related proteins ²⁶. Further, miRNA manipulations often produce modest changes in gene expression and hardly detectable phenotype in normal physiological conditions, but which become apparent in suboptimal conditions ^{43,44}.

Our finding that miR-183/96/182 overexpression improves memory does not conform with a previous report showing that miR-182 is reduced in the lateral amygdala after auditory fear conditioning, and its overexpression impairs long-term auditory fear memory ²⁰. The reason for these different results is unknown but it could be that miR-183/96/182 or miR-182 in the cluster is differently regulated in different brain areas, and/or acts on different targets.

The present results are also highly relevant to neuropsychiatric disorders since both HDAC9 and miR-182 have been implicated in schizophrenia. HDAC9 is one of a few genes with rare copy number variation in schizophrenia patients ⁴⁵ and a hemizygous deletion in a small proportion of patients ⁴⁶. Further, disrupted hippocampal miR-182 signaling has been linked to changes in gene expression observed in schizophrenia and other mental illnesses ⁴⁷. A single nucleotide polymorphism in this miRNA is predicted to be among key SNPs linked to the disease ⁴⁸.

Finally, the present results have implications for the epigenetic roles of PP1. PP1 is known to influence histone acetylation by direct association with HDACs ^{9,49} or by crosstalks with some chromatin targets that affect HDACs ⁵⁰. Our results suggest another indirect control of HDACs by PP1 involving miRNAs. Interestingly, inhibiting HDACs induces substantial upregulation of miR-

183/96/182 cluster in neuroblastoma cell lines ⁵¹, suggesting a possible feedback regulatory loop between HDACs and miRNAs .

2.5. Materials and methods

Animals

To inhibit nuclear PP1 in the mouse hippocampus, transgenic mice carrying the PP1 binding domain of the nuclear inhibitor of PP1, NIPP1 (NIPP1*) fused to EGFP (NIPP1*-EGFP6) and placed under a tetO promoter, were crossed with mice expressing a reverse tetracycline-controlled transactivator 2 (rtTA2) under the control of a CaMKIIalpha promoter⁹. Conditional expression of NIPP1* in double transgenic mice was achieved by feeding the animals with a diet containing doxycycline (Pelodis®) for 8 days (6mg/g of wet mouse chow). The mice were group-housed (4 mice per cage) under a reverse 12h light/dark cycle (25°C, 55% humidity), with food and water *ad libitum*. Behavioral experiments were carried out on rtTA2/NIPP1*-EGFP6 adult male and control littermates (3-5 month old) of C57Bl/6 mice background, during the animals' dark cycle. All experiments were conducted by experimenters blind to genotype. Experiments and animal maintenance were conducted in compliance with the Federation of Swiss Cantonal Veterinary Office and approved by Zürich Cantonal Veterinary Office (54/2012).

Object recognition task

Object recognition training was conducted in a rectangular arena (60cmx50cmx45cm), with gray, opaque walls and translucent plexiglass bottom, under which an infrared light source was placed. It was located in a dedicated behavioral room illuminated by a dim light. Before NOR training, each animal was handled daily for 4 minutes on 4 consecutive days. Then an open field test was conducted by placing each mouse in an empty arena for 10 minutes and measuring the overall locomotion activity. An additional habituation to the empty arena was conducted one day later, before training started. For training (acquisition), three different unfamiliar objects were placed in the center of the arena in a triangular arrangement. Each animal was allowed to explore the objects for five 5-min sessions spaced by a 5-min interval (strong protocol), or a single 10-min session (weak protocol). Object memory was tested in a 5-min session for which one of the familiar objects was replaced with a novel object. For the strong protocol, object memory was tested 24hr after training, and for the weak protocol, memory was tested 24hr (test1) and 48hr (test2) after training. The time that an animal spent exploring

Virus vector design and production

Plasmids for miRNA overexpression and sponge. The scAAV2-EF1a-pri-miR-183/96/182-GFP construct was produced by cutting scAAV2-MCS (Cell Biolabs) by Ball/NotI (New England Biolabs). The transgene cassette containing Ef1a promoter (sequence from pEGP-mmu-miR-182 plasmid; Cell Biolabs), engineered truncated (T)-pri-miR-183/96/182, EGFP (from pEGP-mmu-miR-182 plasmid; Cell Biolabs), WPRE motif and 5'-Ball and 3'-NotI adapters was chemically synthesized by GENEWIZ (South Plainfield, USA) and cloned into the scAAV backbone. Sequence of T-pri-miR-183/96/182 was as follows with mature miRNAs underlined: 5'cctctgcagggtctgcaggctggagagtgtgactcctgtcctgtgtatggcactggtagaattcactgtgaacagtctcagtcagtgaattaccgaagggccataaacagagcagagacagatccgcgagcacccttgagctcctcaccctttctgcctagacctctgtttccaggggtgccagggtacaaagacctcctctgctccttcccagagggcctgttcagtagcatctgcttgccgattttggcactagcacattttgcttgtgtctctccgctgtgagcaatcatgtgtagtgccaatatgggaaaagcgggctgctgcggccacgttcacctcccccgcatccataataaaaacaagtatgctggaggcctcccaccattttggcaatggtagaactcacaccggtaaggtaatgggacccggtggttctagacttgccaactatggtgtaagtgtgagct. The scAAV2-EF1a-pri-miR-183/96/182-GFP allowed the generation of mature miR-183-5p, miR-96-5p, and miR-182-5p sequences annotated in miRBASE v.20 (www.mirbase.org), whose expression was verified both by RT-qPCR and Northern blot analysis in HEK293 cells transfected with scAAV2-EF1a-pri-miR-183/96/182-GFP plasmid (data not shown). We also verified that expression of these miRNAs in HEK293 cells leads to specific repression of reporter mRNAs

bearing miR-183/96/182 sites (data not shown). Control scAAV2-EF1a-control-GFP construct contained fragment of beta-globin intron (sequence from pEGP-mmu-miR-182 plasmid; Cell Biolabs) of length corresponding to T-pri-miR-183/96/182.

To suppress the level of endogenous miRNAs, a triple sponge for the miR-183/96/182 cluster containing 4 binding sites for each miRNA (perfectly complementary and containing a bulge) separated by a 15nt spacer sequence were prepared as described previously²¹. The sponge sequences were assembled in pJ341 plasmids, excised at Hind III sites, and cloned into a p56 plasmid upstream of EGFP ORG. Sequence integrity was verified with sequencing.

scAAV production. Self-complementary AAV production was performed by triple transfection of HEK 293T cells using polyethylenimine with a plasmid bearing the target sequences between the internal terminal repeats of scAAV2, the AAV-helper plasmid encoding Rep2 and Cap for serotype 8, and the pHGTI-Adeno1 plasmid harboring helper adenoviral genes (both kindly provided by C Cepko, Harvard Medical School, Boston, MA, USA). Vectors were purified using a discontinuous iodixanol gradient (Sigma, Optiprep). Encapsidated DNA was quantified by TaqMan RT-PCR following denaturation of the AAV particles by Proteinase K, and titers were calculated as genome copies (GC) per ml.

Stereotaxic surgery and intrahippocampal injection

To overexpress or inhibit miRNAs, virus vectors were prepared as described above. To interfere with miR-182-HDAC9 binding, custom designed miRCURY LNATM microRNA Power Target Site Blockers were obtained from Exiqon. The sequences are: miR-182/HDAC9 TSB: TTTGGCAAAAGTGCTA; negative control TSB: ACGTCTATACGCCCA. The oligonucleotides were stereotaxically injected in to the CA1 region of the hippocampus at a concentration of 1ug/ul, in TurboFect in vivo transfection reagent (Dharmacon). To carry out injections, animals were anaesthetized with 3% isoflurane (AttaneTM) and placed in a stereotaxic frame. Anesthesia was maintained with 1.5% isoflurane in 100% oxygen for throughout surgery. Injection was carried out by lowering a glass

pipette (Blaubrand®, cut to a 20µm inner diameter) filled with virus/oligonucleotides and attached to an injection pump (Stoelting) through a predrilled hole at the following coordinate targeting CA1 region of the hippocampus (from Bregma): *AP*: -2.0mm, *ML*: +/- 1.5mm, *DV*: -1.6mm. A total of 1µl (10⁹GC/ml) of virus vector or 1.5µl of TSB oligos was injected into each hippocampus at a rate of 0.2µl/min. The glass pipette was left in place for an additional 5 minutes, before it was carefully withdrawn and the wound was closed. The animals were allowed to recover for up to 2 weeks in their home cage before behavioral testing.

Cell culture

Mouse neuroblastoma (N2A) cells were obtained from American Type Cell Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle's Medium (DMEM- high glucose), supplemented with 10%(v/v) FBS (Gibco®) and 1% Antibiotic-Antimycotic (Gibco®). These cells were chosen for their fast-growth, easy maintenance and transfection, and potential for neuronal differentiation making them a convenient model for studying miRNA biogenesis. The cells were proven free of mycoplasma contamination through regular tests. Before the start of experiments, the cells were passaged 1:4 and split every 3 days for at least 5 passages. On transfection day, 150,000 - 300,000 cells were plated in 6-well plates. Transfection of a pool of siRNAs targeting PP1γ 3'UTR (ThermoFischer Scientific, sequences in supplementary material) or negative control siRNA (All Star negative control, Qiagen) was carried out with HiPerfect® transfection reagent (Qiagen). In pri-miRNA processing assays, inhibition of nuclear PP1 was achieved by overexpression of a plasmid containing NIPP1 construct (Origene). Then the cells were returned to the incubator and allowed to grow for 72 hours prior to harvest or further treatment. In KCl treatment conditions, 50mM of KCl was added to plated cells 1hr before harvest (unless indicated otherwise). In actinomycin D (Tocris®) treatment conditions, the cells were treated with 5mg/ml of the drug prepared in DMSO 1hr before KCl treatment or harvest. Rescue of PP1γ knockdown effects was performed by co-transfecting a plasmid containing PP1γ open reading frame (Origene) with siRNA against PP1γ 3'UTR. The cells were harvested by removing the medium, washing with ice-cold PBS 3 times, and lysing with Tri-reagent® (for RNA extraction) or RIPA buffer (for protein extraction). All

experiments were conducted on at least three replicates from different passage number, and repeated at least three times.

Luciferase-based pri-miRNA processing and HDAC9 target validation assays

Pri-miRNA processing assays were conducted as previously described²⁴. The assay quantifies Drosha processing of pri-miRNA based on the decrease in luciferase activity, which is inversely proportional to the Drosha processing of pri-miRNA. Briefly, fragments of pri-mir-182 and pri-mir-183 containing the hairpin and 100 bp flanking sequence were amplified from genomic DNA. The PCR products were digested with the respective restriction enzymes and inserted at MCS in pmirGLO vector (Dual Reporter Luciferase Assay System, Promega) downstream to firefly luciferase reporter. Cropping of the hairpin stem-loop of the inserts result in destabilization of the firefly reporter resulting in decrease in firefly luminescence. The unperturbed Renilla reporter produces stable luminescence, which serves as internal normalization control. Dual-luciferase reporters with pri-mir-182, and pri-mir-183 were transfected in N2a cells using cationic liposomes (Lipofectamine 2000 reagent, Invitrogen). PP1 manipulations were performed by simultaneously transfecting N2a cells with NIPP1 over-expressing plasmid (Origene). The cells were lysed 48 hr post-transfection with passive lysis buffer (Promega) treatment at room temperature for 10 minutes. The lysates were then transferred to a 96 well plate, and luciferase activities of firefly and Renilla were read through luminometer GloMax 96 (Promega) equipped with dual injections dispersing LAR II (for firefly luciferase quantification) and Glomax (for renilla luciferase quantification) reagents sequentially.

For validation of HDAC9 targeting by miR-182, the same vector system and cloning strategy as described above was used (with the exception that HDAC9 3'UTR sequence containing miR-182 target site was amplified from genomic DNA using the following primers and inserted into the vector: *hdac9_F1_NheI*-GGCATAgctagcAGGATATGTGCCAGGCAGTC, *hdac9_R1_Sall*-CGCTTAgtcgacAATGGGCGTCATTGTTCTTC). Dual-reporter vectors with 3'UTR HDAC9 inserts were transfected in N2a cells using cationic liposomes (Lipofectamine 2000 reagent, Invitrogen). MiR-182 mimic (Qiagen) was

simultaneously transfected to the cells. The cells were lysed 24 hr post-transfection with passive lysis buffer (Promega) treatment at RT for 10 minutes. The lysates were then transferred to a 96-well plate, and luciferase activity of firefly and Renilla were read through luminometer GloMax 96 (Promega) equipped with dual injections dispersing LAR II (for firefly luciferase quantification) and Glomax (for renilla luciferase quantification) reagents sequentially.

RNA extraction and RT-qPCR

Mouse hippocampal tissue was homogenized using TissueLyser (Qiagen) in Trizol® reagent (Invitrogen). Total RNA was extracted by phenol-chloroform precipitation. For extraction from cells, the medium was removed, the cells were washed three times with ice cold-PBS, lysed and homogenized by adding Trizol® to the plates. Subcellular fractionation of nuclear and cytoplasmic RNA was performed using Norgen's Cytoplasmic and nuclear RNA purification kit (Norge BioTek, Canada). One microgram of total RNA was treated with RNase free DNase (Promega) and reverse transcribed using miScript II RT kit® (Qiagen). Miscript primer assays for mature and precursor miRNAs were used to amplify the respective transcripts from a cDNA pool (Supplementary Table 1). For mRNA quantification, custom designed gene specific primers were used (Supplementary Table 1). Real time PCR was performed on LightCycler 480® (Roche). Small nuclear RNA (RNU6) or GAPDH was used as endogenous control and quantification was performed as previously⁵².

Deep sequencing

The quality and quantity of RNA was determined using a Bioanalyzer® (Agilent) and Qubit® fluorometer (Invitrogen) respectively. Small RNA libraries were prepared from 1ug of total RNA using TruSeq® Small RNA Kit (Illumina) according to manufacturer's instructions. Briefly, 3' and 5' adapters were ligated to small RNAs using T4 DNA ligase. Ligated RNA was reverse transcribed to cDNA using superscript II reverse transcriptase. The resulting cDNA template was amplified by PCR to generate a cDNA library. For each sample, a common forward primer (which binds to the 5' adapter complement) and a unique reverse primer (which binds to the 3' adapter complement and contains a unique sequence for each sample) were used. The quality of the

resulting amplicons was analyzed on a high sensitivity DNA ChIP (Bioanalyzer). Next, the cDNA construct was run on a 6% PAGE gel and amplicons corresponding to adapter ligated miRNA sequences (145-160nt) were excised out. DNA from excised gel was eluted and precipitated in Ethanol. The size, purity, and amount of cDNA was assessed on high sensitivity DNA ChIP (Bioanalyzer). Then a titration run was done to check quality of the library and validate the amount. Finally, multiplexed samples were sequenced on Hi-Seq 2000 using TrueSeq™ SBM v5 sequencing kit. The resulting sequences were demultiplexed and sorted to individual samples according to their index codes. The error rate for each library was estimated based on a PhiX reference spiked before sequencing. Next, adapters were trimmed from the reads and the resulting inserts were categorized by size. Bioinformatic analysis of sequencing reads was performed using ncPRO-seq pipeline⁵³. After confirming that the majority of reads are attributed to the size range of miRNAs, the sequences were mapped and aligned to the mouse reference genome (NCBI37/mm9) using bowtie algorithm. The number of reads uniquely mapping to miRNAs were normalized to the total number of reads in each sample. Finally, differential expression analysis of miRNAs regulated upon expression of NIPP1* and/or during memory formation was performed using Wilcoxon unpaired test⁵⁴.

Protein extraction and Western blot

Total protein was extracted from N2A cells using radio immunoprecipitation buffer (RIPA) with 1:1000 protease inhibitor cocktail (Sigma-Aldrich) and 1:500 phenyl methyl sulfonyl fluoride (PMSF). Cells were lysed directly on the culture plate with 100-150 ul RIPA and scraped off with a cell scraper. The lysate was transferred to a micro centrifuge tube and sonicated for 5 cycles, each comprising 30 seconds of sonication with 30 seconds intervals. The resulting mixture was centrifuged for 15 minutes at 14,000g to separate the protein mixture (supernatant) from cellular debris (pellet). 20-40 ug proteins were resolved on SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-rad). Membranes were blocked in 3% BSA for one hour, and then incubated in primary (overnight at 4°C) and secondary (one hour at room temperature) antibodies. They were scanned using Odessey IR scanner (Li-Cor Bioscience), and band intensity was determined and quantified using image analysis

software (ImageJ). The following antibodies were used: primary – HDAC9 (Abcam, ab59718), PP1 γ (Millipore, 07-1218); secondary – anti-mouse IRDye® goat anti-mouse (LI-COR, 925-32210) and IRDye® goat anti-rabbit (LI-COR, 925-32211).

Statistical analyses

For deep-sequencing data, differential expression between two groups was assessed by Wilcoxon signed-rank test. For miRNA and mRNA qPCRs and behavioral experiments with two groups, two-tailed Student *t* test was used to assess statistical significance. For other qPCR experiments with more than 2 groups, ANOVA was used. Tukey's or least significant difference (LSD) *post hoc* analyses were conducted when appropriate. Outliers were defined as values beyond two standard deviation from a group mean and were removed from the analysis. Significance was set at $p \leq 0.05$ for all tests and two-sided tests were performed. Statistical analysis was performed using GraphPad prism or R statistical software.

2.6. Acknowledgments

This work was supported by the University of Zurich, the Swiss Federal Institute of Technology, the Swiss National Science Foundation and the National Competence Center for Research "Neural plasticity and Repair". We thank Yannick Rothacher, Cécile Hauser, Jennifer Brown, and Lubka Spassova for technical help, Jean-Claude Paterna for virus production, Gregor Fisher and animal caretakers for mouse colony management, Witold Filipowicz and Olivier Voinnet for constructive discussion and critical reading of the manuscript.

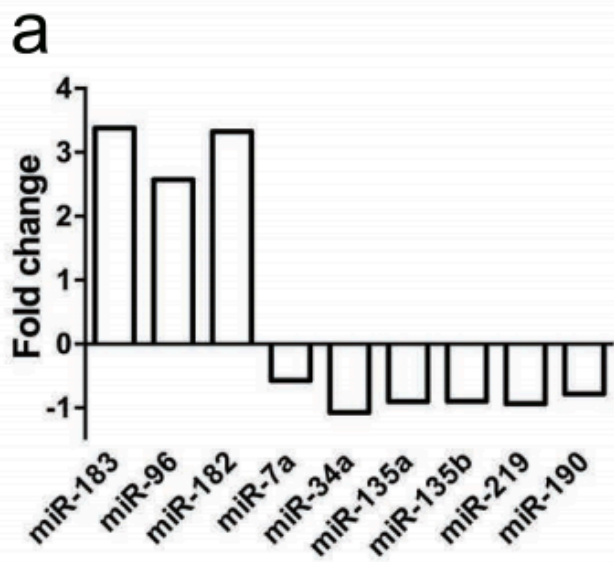
2.7. Authors' contribution

B.T.W. and I.M.M initiated and designed the study, analyzed the results and wrote the manuscript. B.T.W. performed behavioral experiments and stereotaxic injections, sequencing, and did molecular analysis of brain samples. A.J designed and executed *in vitro* experiments, and helped in the interpretation and drafting of *in vitro* data. N.G and A.J conducted RT-PCR experiments for *in vivo* miRNA target validation and miRNA expression after weak memory protocol respectively. E.A.K designed cloning experiments and

conducted them with A.J. J.K. produced virus vectors and constructs for miRNA overexpression and sponge. A.M. analyzed sequencing data.

2.8. Figures & Figure Legends

PP1 inhibition



NOR training

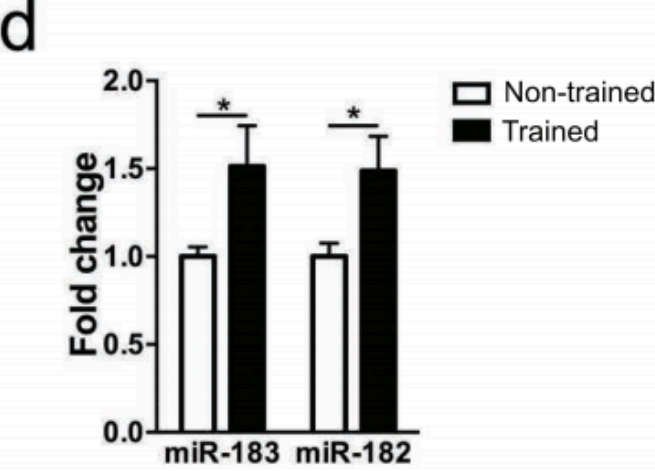
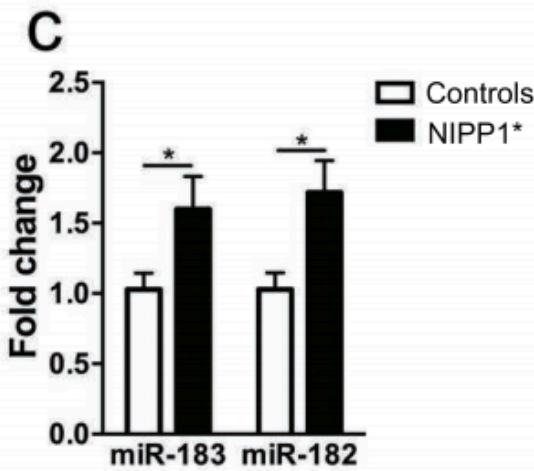
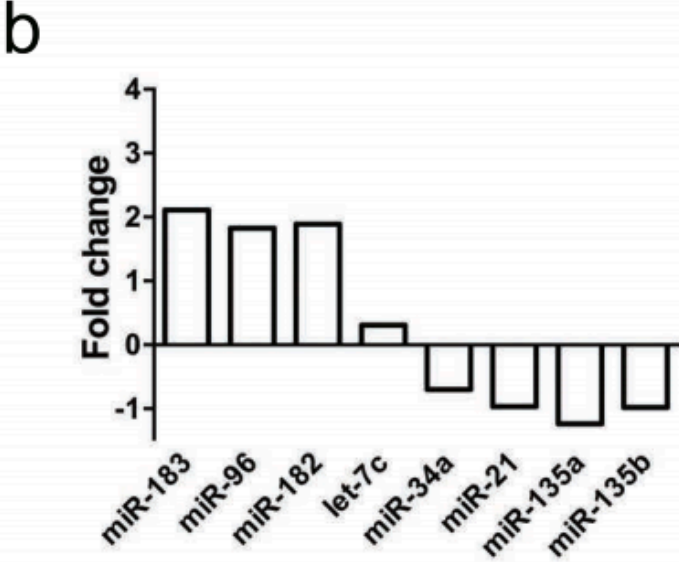


Figure 1: PP1 inhibition and NOR training induce differential expression of several miRNAs in the mouse hippocampus. NIPP1* and control mice were trained on an NOR task (Trained) or exposed to the training chamber alone (Non-trained). Deep sequencing was conducted on pooled hippocampal samples (n=6 in each group) collected 30min after the end of training. Several miRNAs are differentially expressed (adjusted $p < 0.05$) in non-trained NIPP1* mice compared to non-trained controls (a, expressed as log2 of fold change), and in trained controls compared to non-trained controls (b, expressed as log2 of fold change). Some of the miRNAs identified by deep sequencing were similarly upregulated in an independent set of experiments in both non-trained NIPP1* mice (c, miR-183: controls, n=11; NIPP1*, n=11; $t_{20}=2.19$, $*p < 0.05$; miR-182: controls, n=11; NIPP1*, n=11; $t_{20}=2.68$, $*p < 0.05$); and control mice trained on NOR (d; miR-183: non-trained, n=12; trained, n=13; $t_{23}=2.07$, $*p = 0.05$; miR-182: non-trained, n=13; trained, n=13; $t_{24}=2.32$, $*p < 0.05$). Bar graphs represent mean \pm s.e.m.

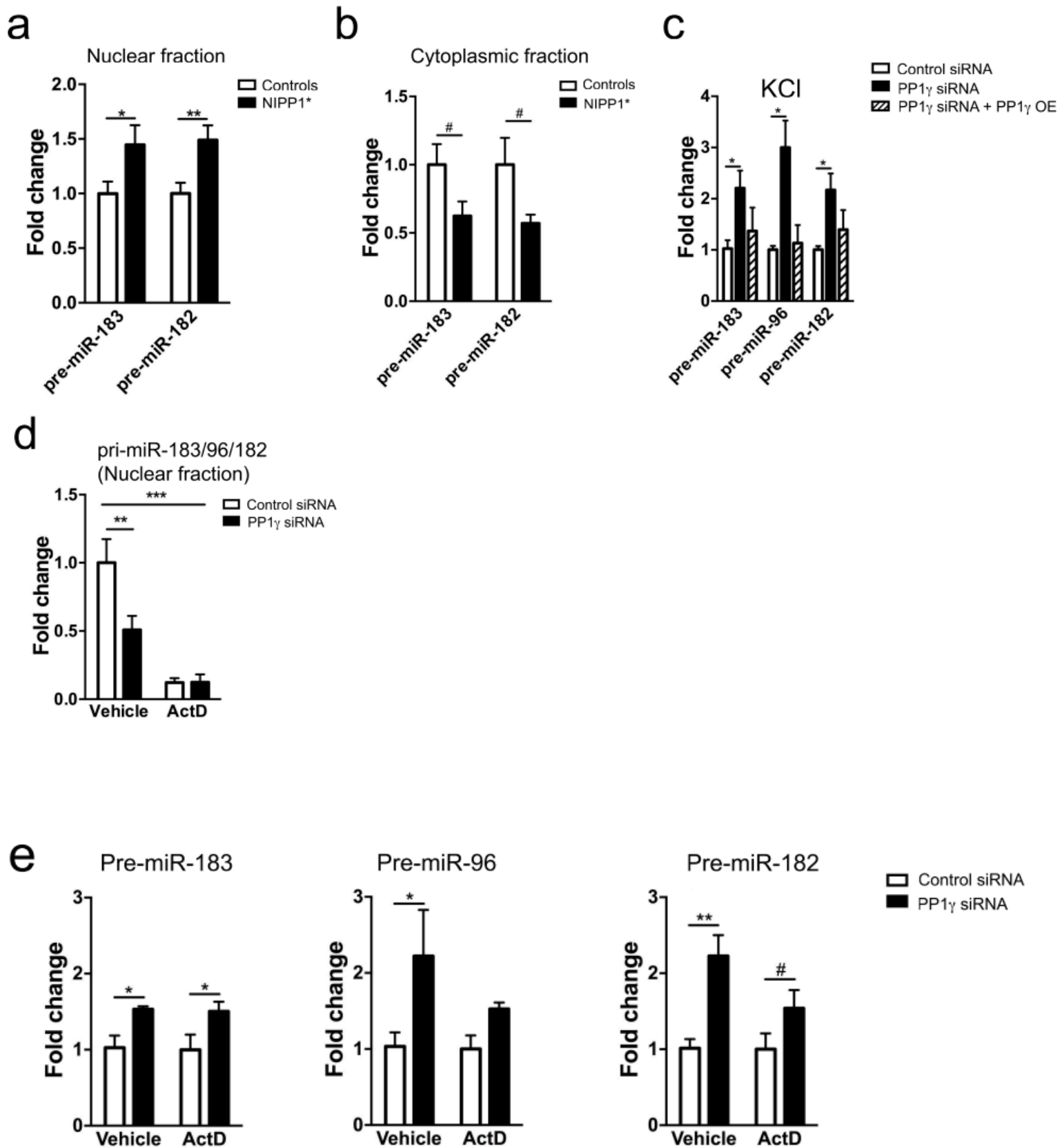


Figure 2: PP1 inhibition up-regulates pre-miR-183/96/182 expression. (a) Pre-miR-183/96/182 expression in the nuclear fraction of the hippocampus from NIPP1* and control mice (pre-miR-183: controls, n=11; NIPP1*, n=11; $t_{20}=2.12$, * $p<0.05$; pre-miR-182: controls, n=11; NIPP1*, n=11; $t_{20}=2.92$, ** $p<0.01$). (b) Cytoplasmic pre-miR-183/96/182 level in the hippocampus of NIPP1* mice and control littermates (pre-miR-183: $t_{10}=2.05$, # $p<0.1$; pre-miR-182: $t_{10}=2.09$, $p<0.1$; controls, n=6; NIPP1, n=6). (c) PP1 γ knockdown combined with KCl stimulation (1hr) causes upregulation of pre-miR-183, pre-miR-96, and pre-miR-182, which is reversed by PP1 γ overexpression (pre-miR-183: one-way ANOVA, $F_{2,6}=3.123$, $p=0.12$; t-test: $t(4)=3.089$, * $p=0.05$; pre-miR-96: one-way ANOVA, $F_{2,6}=9.19$, $p=0.01$; t-test: $t(4)=3.748$, * $p<0.05$; pre-miR-182: one-way ANOVA, $F_{2,6}=4.13$, t-test: $t(4)=3.521$, * $p<0.05$). (d) Nuclear pri-miRNA regulation by PP1 γ knockdown and the effect of ActD treatment in N2A cells after 1hr of KCl stimulation (Two-way ANOVA, ActD: $F_{1,8}=35.62$, *** $p<0.001$, PP1 γ : $F_{1,8}=5.375$, $p<0.05$, post-hoc: vehicle ** $p<0.01$). (e) ActD treatment of N2A cells does not fully abolish pre-miRNA upregulation induced by PP1 γ knockdown; left panel: pre-miR-183, two-way ANOVA: PP1 γ - $F_{1,8}=12.24$, $p=0.008$ (post-hoc: vehicle $t_8=2.48$, * $p<0.05$; ActD $t_8=2.47$, * $p<0.05$); middle panel: pre-miR-96, two-way ANOVA: PP1 γ - $F_{1,8}=6.643$, $p=0.033$ (post-hoc: vehicle $t_8=2.53$, * $p<0.05$; ActD $t_8=1.12$, $p=0.30$); right panel: pre-miR-182, two-way ANOVA: PP1 γ - $F_{1,8}=15.92$, $p=0.004$ (post-hoc, Vehicle $t_8=3.91$, ** $p<0.01$; ActD $t_8=1.74$, # $p=0.1$). Bar graphs represent mean \pm s.e.m.

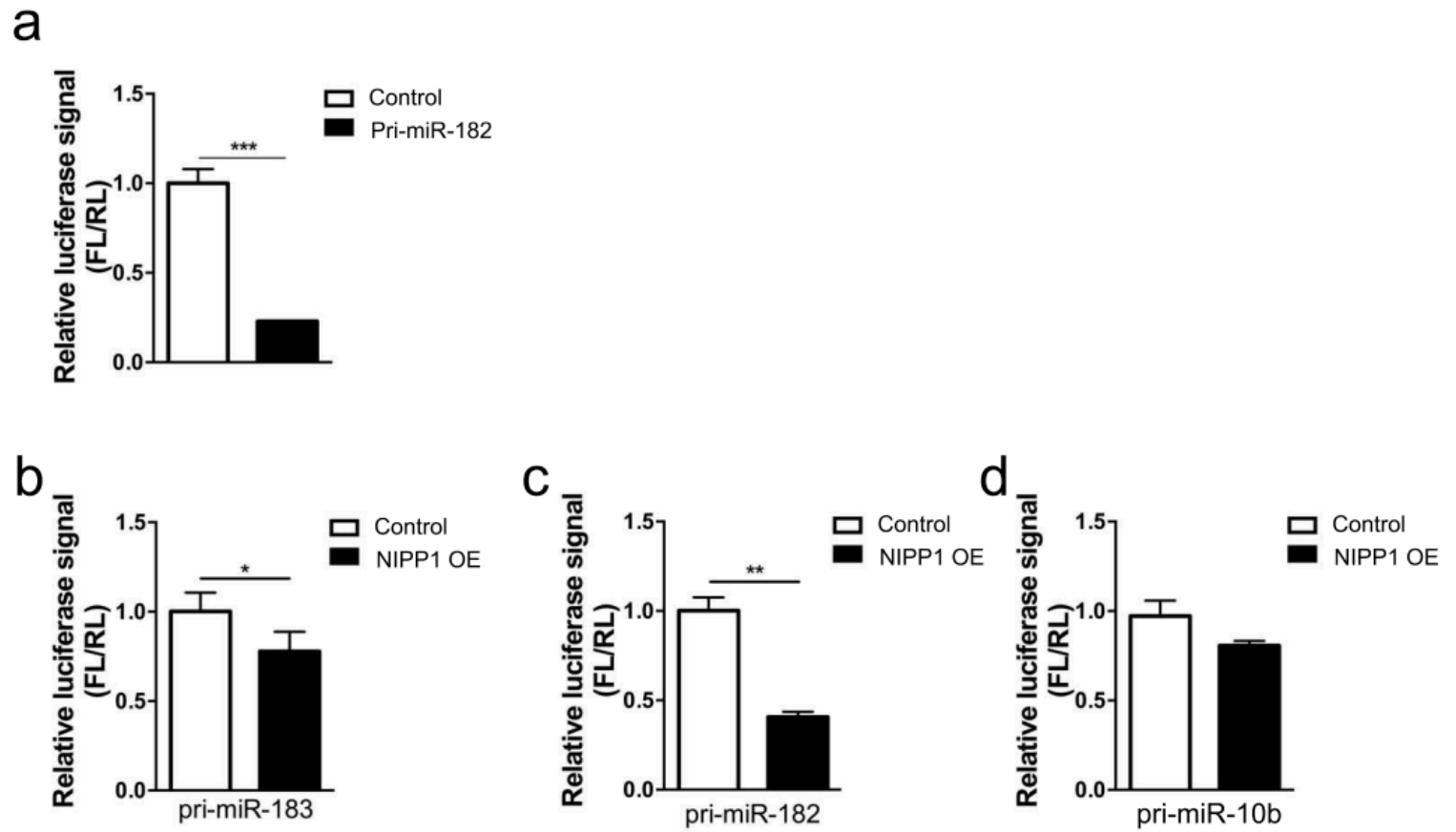


Figure 3: Analyses of pri-miRNA processing. (a) Relative signal of firefly luciferase (FL) to renilla luciferase (RL) is reduced in pmirGLO_pri-miR-182 transfected cells (Pri-miR-182) compared to control cells transfected with an empty vector (Control) ($t_4=9.8$, $p<0.001$). *** $p<0.001$. (b-d) Overexpression of a nuclear inhibitor of PP1 (NIPP1 OE) reduces FL/RL signal in cells expressing pri-miR-183 (b, $t_5=2.71$, $*p=0.04$) and pri-miR-182 (c, $t_4=7.45$, $**p<0.001$), but not pri-miR-10b (d, $t_4=1.83$, $p=0.14$). Bar graphs represent mean \pm s.e.m.

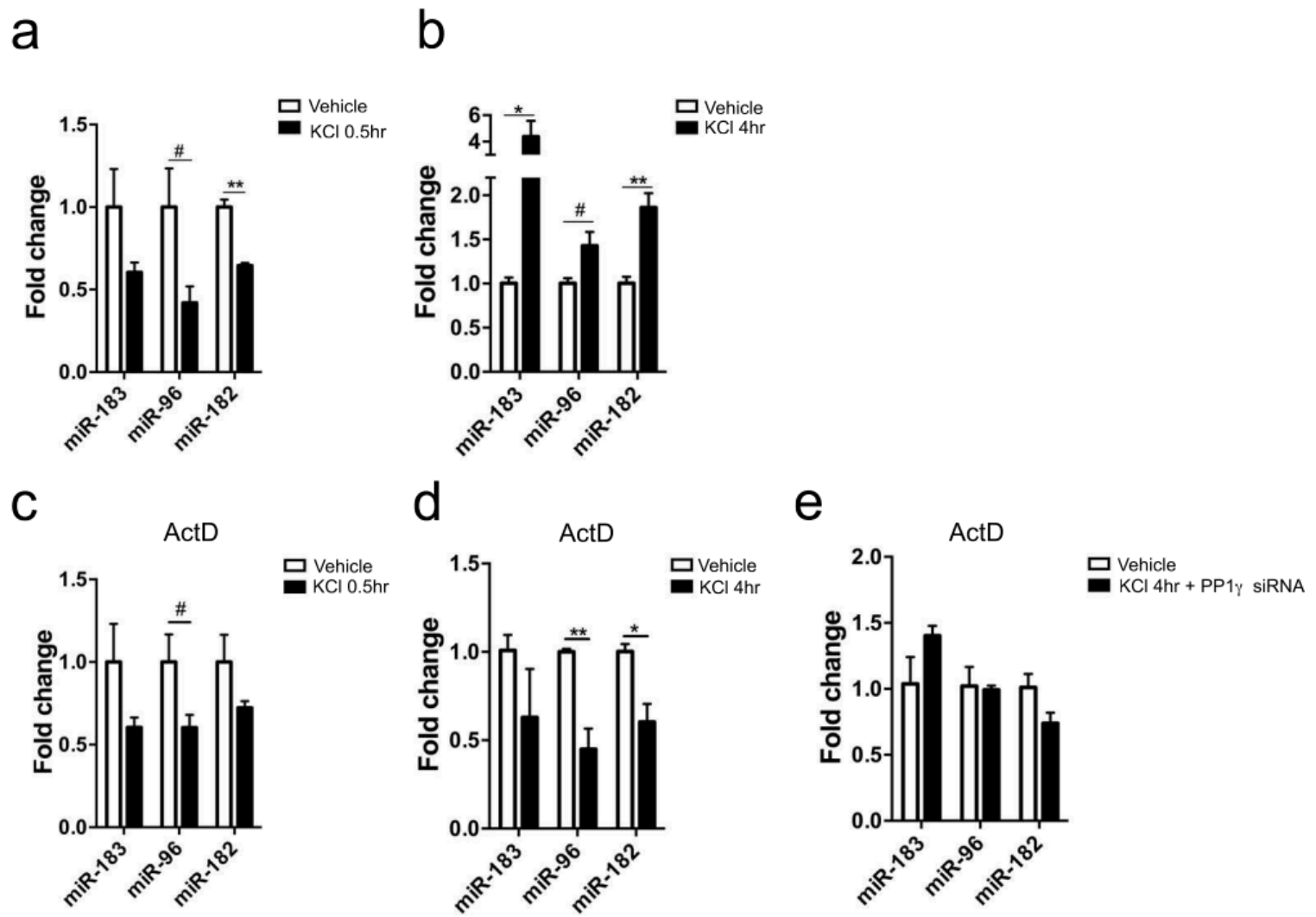


Figure 4: PP1 inhibition facilitates an increase in mature miRNAs following neuronal stimulation. (a) Mature miRNAs level is lower 0.5hr after KCl treatment (miR-183: $t=1.66$, $p=0.17$; miR-96: $t=2.28$, $\#p<0.1$; miR-182: $t=7.2$, $**p<0.01$). (b) Mature miRNAs level is increased 4hr after KCl treatment (miR-183: $t=2.81$, $*p<0.05$; miR-96, $t=2.55$, $\#p<0.1$; miR-182, $t=4.86$, $**p<0.01$). (c) Effect of Actinomycin D (ActD) treatment and 0.5hr of KCl stimulation on mature miRNA level (miR-183, $t=1.66$, $p=0.17$; miR-96, $t=2.17$, $\#p<0.1$; miR-182, $t=1.66$, $p=0.18$). (d) ActD treatment reduces the level of mature miRNAs 4hr after KCl stimulation (miR-183, $t=1.32$, $p=0.26$; miR-96, $t=4.68$, $**p<0.01$; miR-182, $t=3.59$, $*p<0.05$). This effect of ActD is reversed by PP1 γ knockdown (e: miR-183, $t=1.68$, $p=0.17$; miR-96, $t=0.20$, $p=0.85$; miR-182, $t=2.09$, $p=0.10$). Bar graphs represent mean \pm s.e.m.

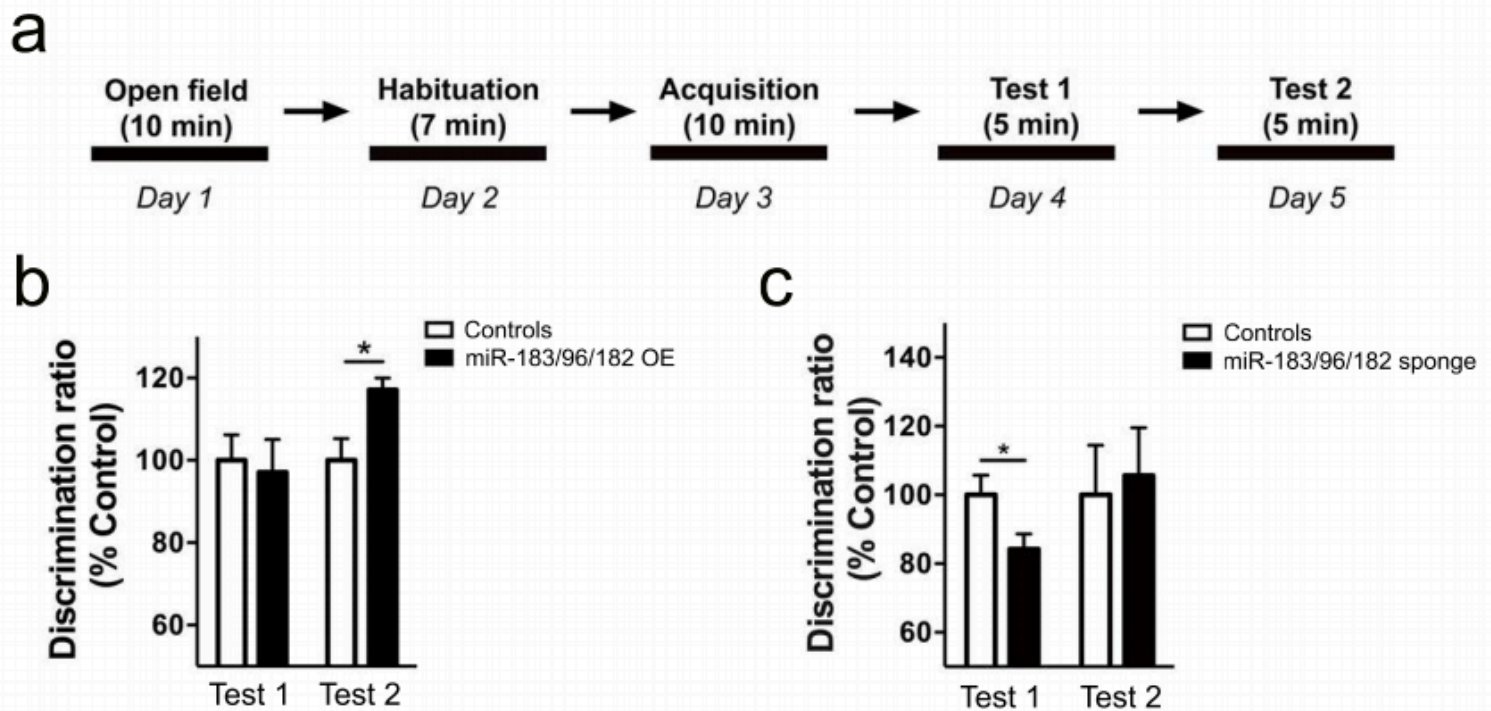


Figure 5: MiR-183/96/182 modulation in the hippocampus affects long-term object memory. (a) Experimental setup. (b) Novel object discrimination ratio expressed as percent of controls) in miR-183/96/182 overexpressing mice and controls during memory test 24hr (Test 1, $t_{32}=0.298$, $p=0.77$) and 48hr (Test 2, $t_{32}=2.65$, $*p<0.05$) after acquisition. Controls, $n=19$; miR-183/96/182, $n=15$. (c) Novel object discrimination ratio (expressed as percent of controls) in miR-183/96/182 sponge expressing mice and controls during memory test 24hr (Test 1, $t_{18}=2.18$, $*p=0.043$) and 48hr (Test 2, $t_{16}=1.079$, $p=0.297$); controls, $n=9-10$, miR-183/96/182 sponge, $n=10-11$. Bar graphs represent mean \pm s.e.m.

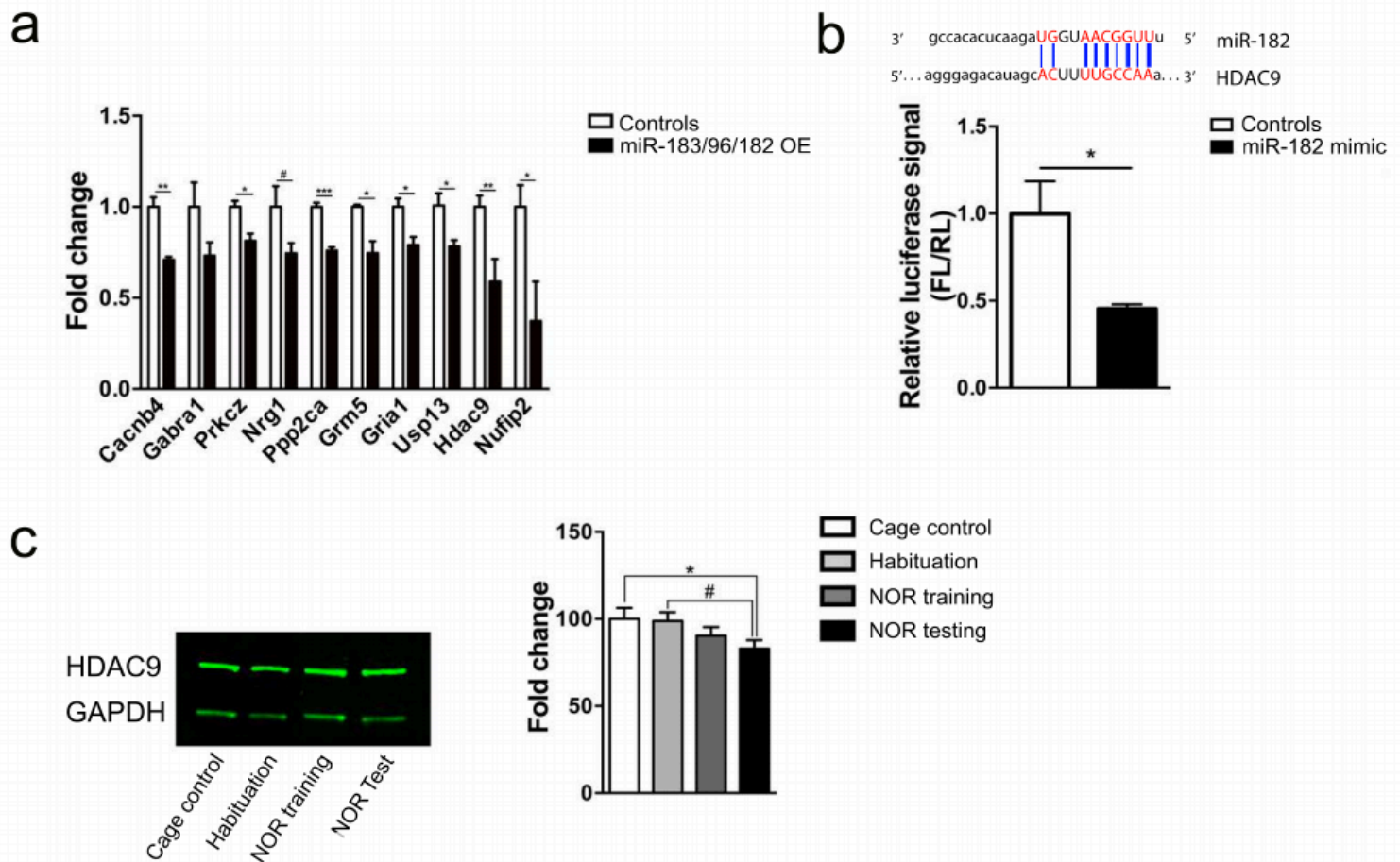


Figure 6: MiR-183/96/182 cluster decreases the expression of genes involved in plasticity and altered by NOR training, including HDAC9. (a) Expression level of target genes in the hippocampus of miR-183/96/182 overexpressing mice: *Cacnb4* ($t_6=5.42$, $**p<0.01$), *Gabra1* ($t_6=1.768$, $p=0.127$), *Prkcz* ($t_6=3.675$, $*p=0.01$), *Nrg1* ($t_5=2.212$, $\#p=0.08$), *Ppp2ca* ($t_6=8.159$, $***p<0.001$), *Grm5* ($t_5=3.246$, $*p=0.02$), *Gria1* ($t_6=3.261$, $*p=0.02$), *Usp13* ($t_6=2.98$, $*p=0.02$), *Hdac9* ($t_{10}=3.25$, $*p<0.01$), and *Nufip2* ($t_{10}=2.74$, $*p<0.05$). Controls, $n=3-7$; miR-183/96/182, $n=4-5$. (b) Top panel: Predicted target site of miR-182 seed sequence on HDAC9 3' UTR. The seed sequence and its corresponding target sequence are highlighted in red (adapted from www.microrna.org); bottom panel: Relative luciferase activity (Firefly luciferase (FL) to Renilla Luciferase (RL)) measured in N2A cells in the presence or absence of miR-182 mimic ($t_7=2.88$, $p<0.05$). (c) Representative blot (left panel) and quantification (right panel) of HDAC9 protein in mouse hippocampal extracts after NOR training (One-way ANOVA: $F_{3,28}=2.06$, $p=0.13$; t-test: cage control vs NOR testing ($t_{14}=2.047$, $\#p<0.1$, habituation vs NOR testing ($t_{13}=2.237$, $*p<0.05$). Bar graphs represent mean \pm s.e.m.

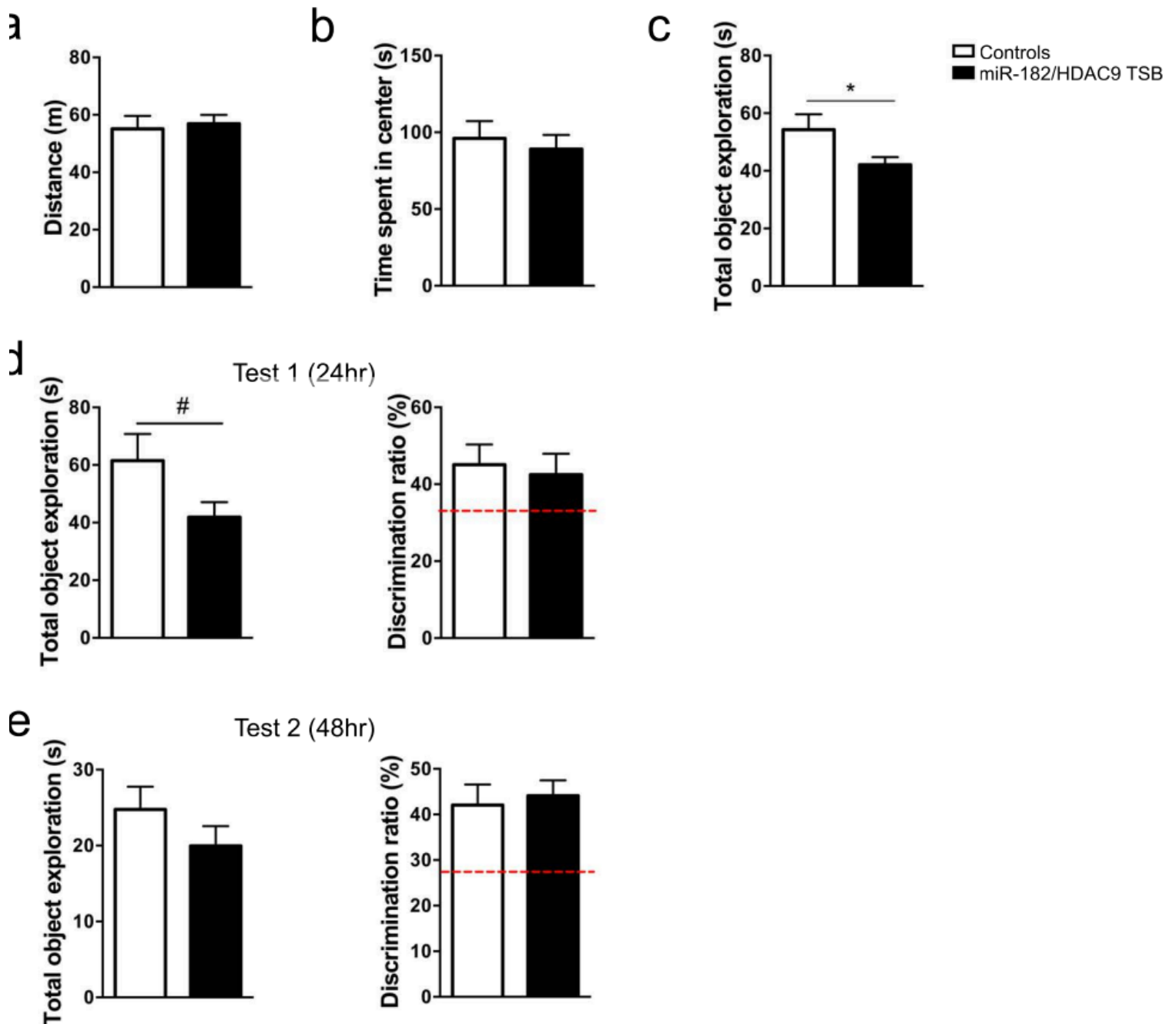
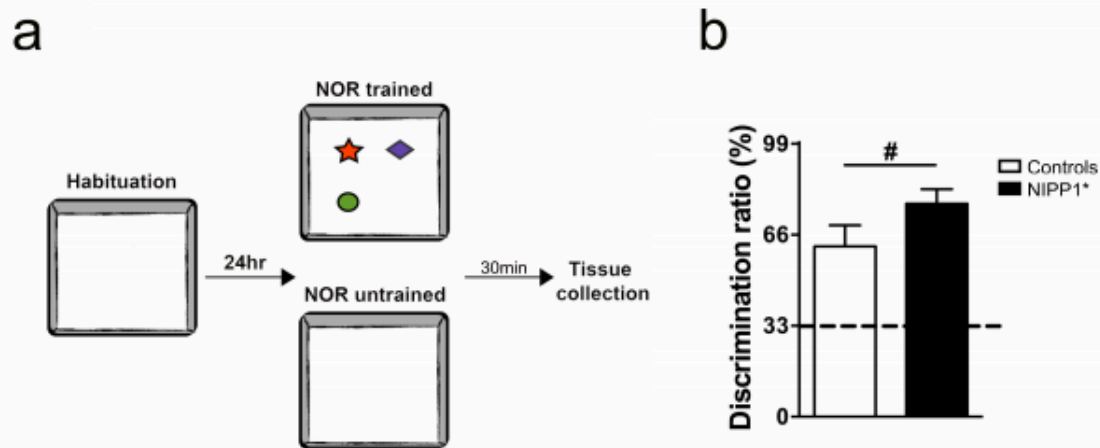
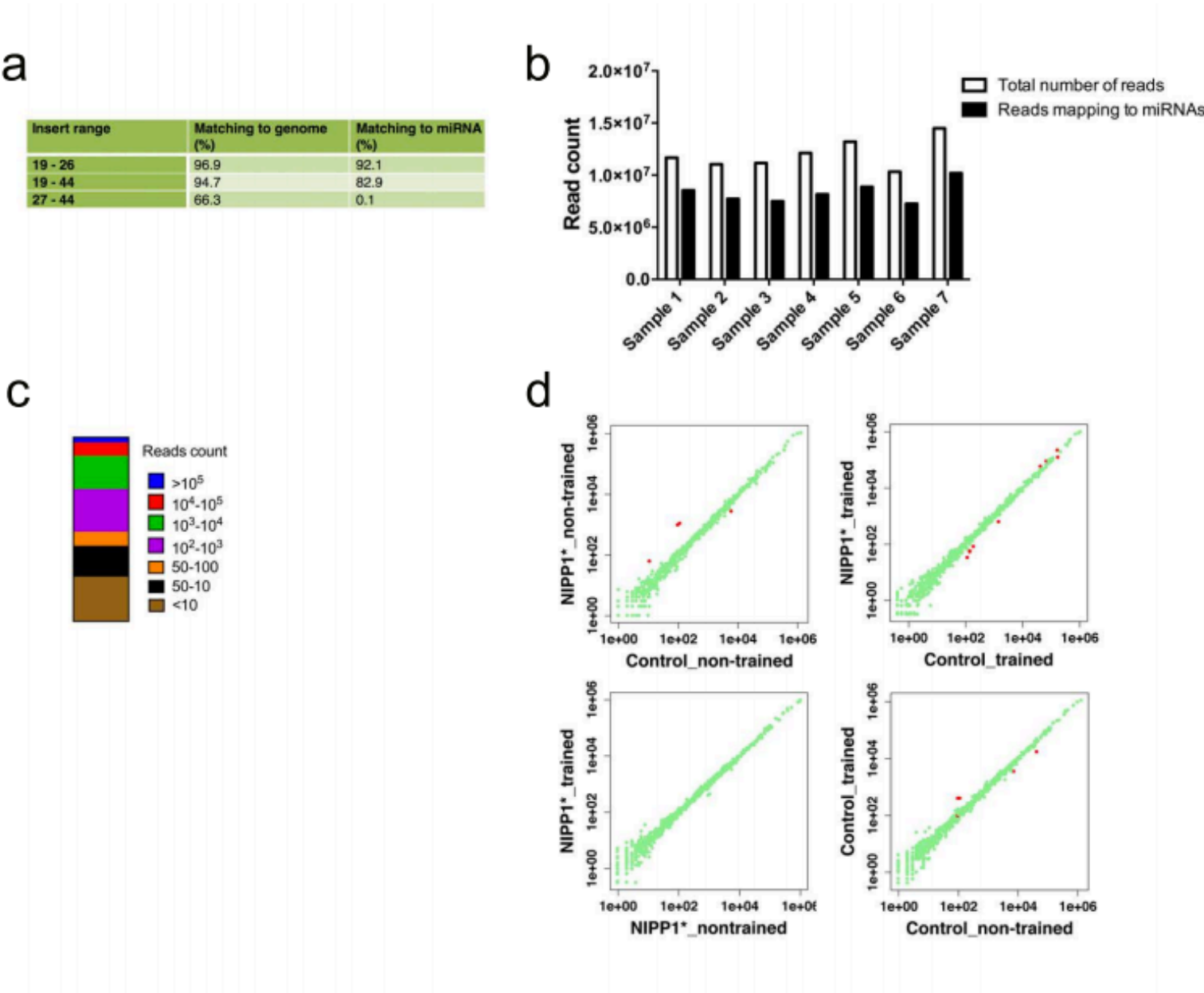


Figure 7: Effect of miR-182/HDAC9 target site blocker (TSB) on NOR performance in mice overexpressing miR-183/96/182. (a-c) Behavioral measures during acquisition: total distance covered (a, $t_{15}=0.333$, $p=0.74$); total time spent in center (b, $t_{15}=0.481$, $p=0.64$); and total time spent exploring the objects (c, $t_{15}=2.12$, $*p=0.05$). (d) Left panel: Total time spent exploring objects during test 1 ($t_{15}=1.90$, $\#p=0.08$). Right panel: discrimination ratio of novel versus familiar objects during test 1 ($t_{15}=0.343$, $p=0.74$). (e) Left panel: total time spent exploring objects during test 2 ($t_{15}=1.22$, $p=0.24$). Right panel: discrimination ratio of novel versus familiar objects during test 2 ($t_{15}=1.04$, $p=0.32$). Controls, $n=9$; miR-182/HDAC9 TSB, $n=8$. Bar graphs present mean \pm s.e.m.

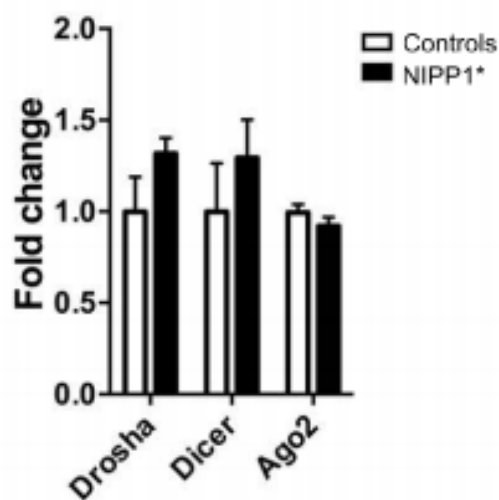
2.9. Supplementary Figures & Table



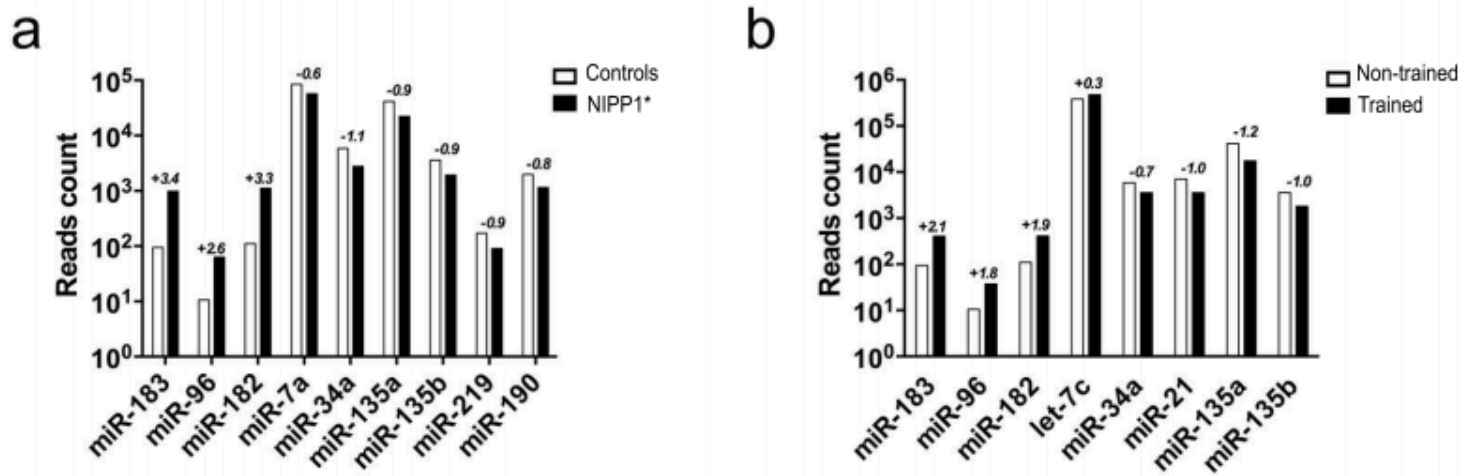
Supplementary Figure 1: NOR training of mice before deep sequencing screening. (a) Experimental set-up for NOR training conducted prior to deep sequencing. (b) Performance of animals used for sequencing tested 5min after training, expressed as discrimination ratio. Both groups demonstrated significant discrimination of novel object (chance level set at 33%) (one-sample t-test, control: $t_5=4.589$, $p<0.01$; NIPP1*: $t_5=8.525$, $p<0.001$) but NIPP1* mice had better performance than controls (unpaired t test between controls and NIPP1*: $t_{10}=1.93$, $\#p=0.08$). Controls, $n=6$; NIPP1*, $n=6$. Bar graphs represent mean \pm s.e.m.



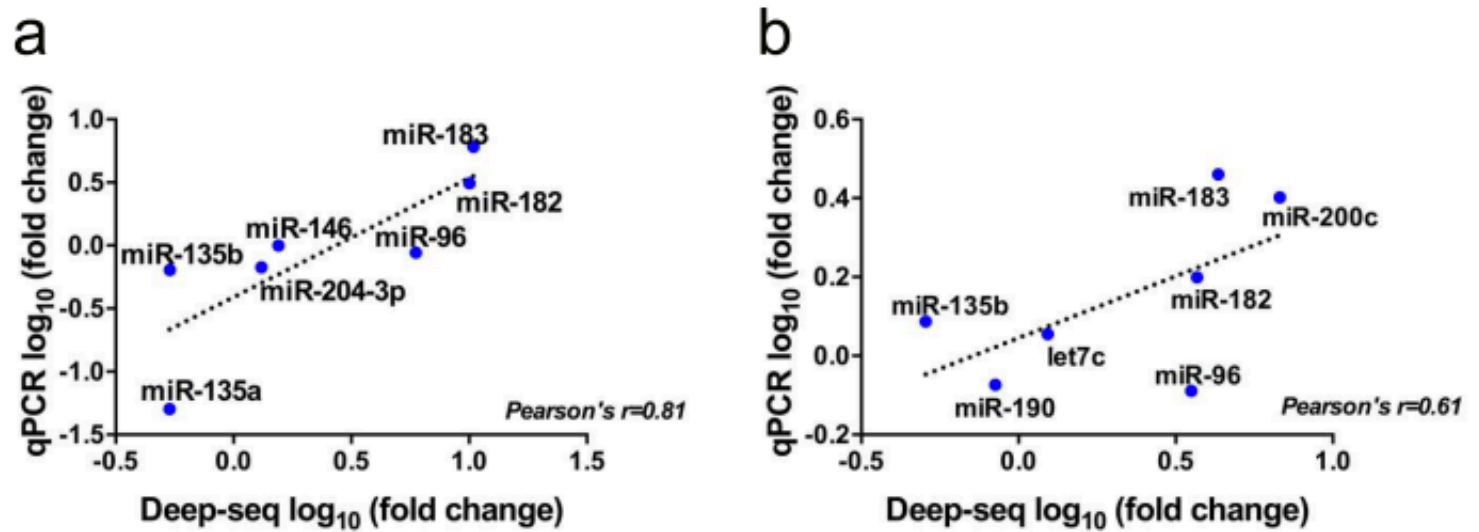
Supplementary Figure 2: Distribution of reads obtained by deep sequencing and correlation between groups. (a) Proportion of sequence reads mapping to the genome and known miRNAs. (b) Comparison of number of reads that uniquely map to miRNAs in each sample (c) Average distribution of all identified miRNAs based on reads count expressed as 'read counts'. (d) Correlation of expression level of all identified miRNAs (each represented as a dot in $\ln(\text{read count})$) between the different experimental groups (NIPP1* and control littermates, trained or untrained). Red dots represent differentially expressed miRNAs (adjusted $p < 0.05$).



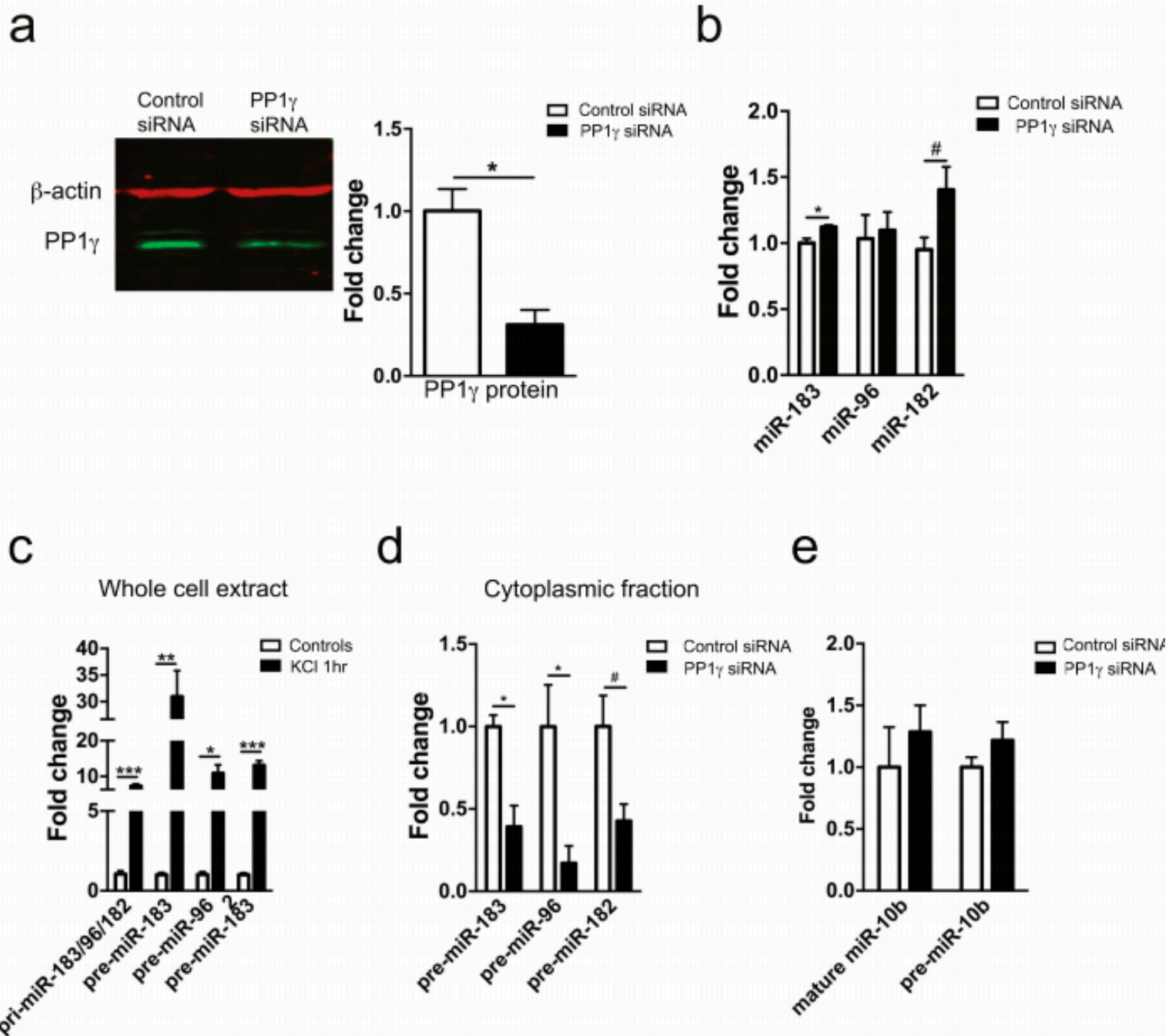
Supplementary Figure 3: qPCR quantification of major components of the miRNA biogenesis machinery in NIPP1* and control mice. Drosha: $t_8=1.54$, $p=0.16$; Dicer: $t_8=0.89$, $p=0.40$; Ago2: $t_8=1.10$, $p=0.30$; Controls, $n=5$; NIPP1*, $n=5$. Bar graphs represent mean \pm s.e.m.



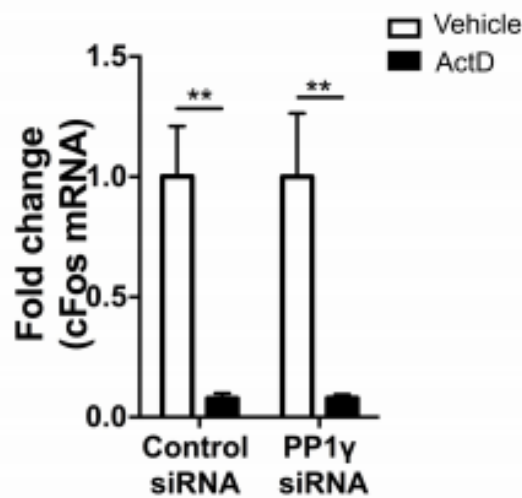
Supplementary Figure 4: Relative abundance of differentially expressed miRNAs based on reads count per million (log 2). (a) NIPP1* compared to control mice; (b) trained controls compared to non-trained controls. The number above bars indicates fold change between groups.



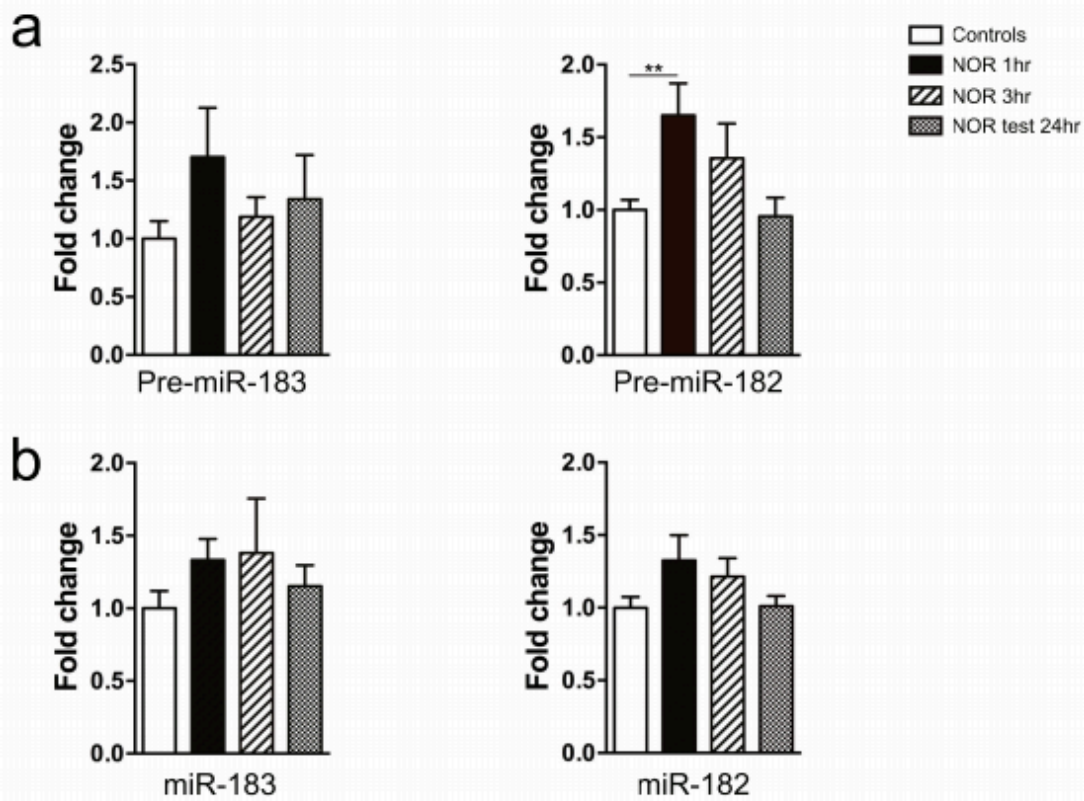
supplementary Figure 5: Correlation between deep sequencing and qPCR-based quantification of miRNAs differentially expressed: (a) in NIPP1* and control mice; (b) in NOR-trained and untrained mice.



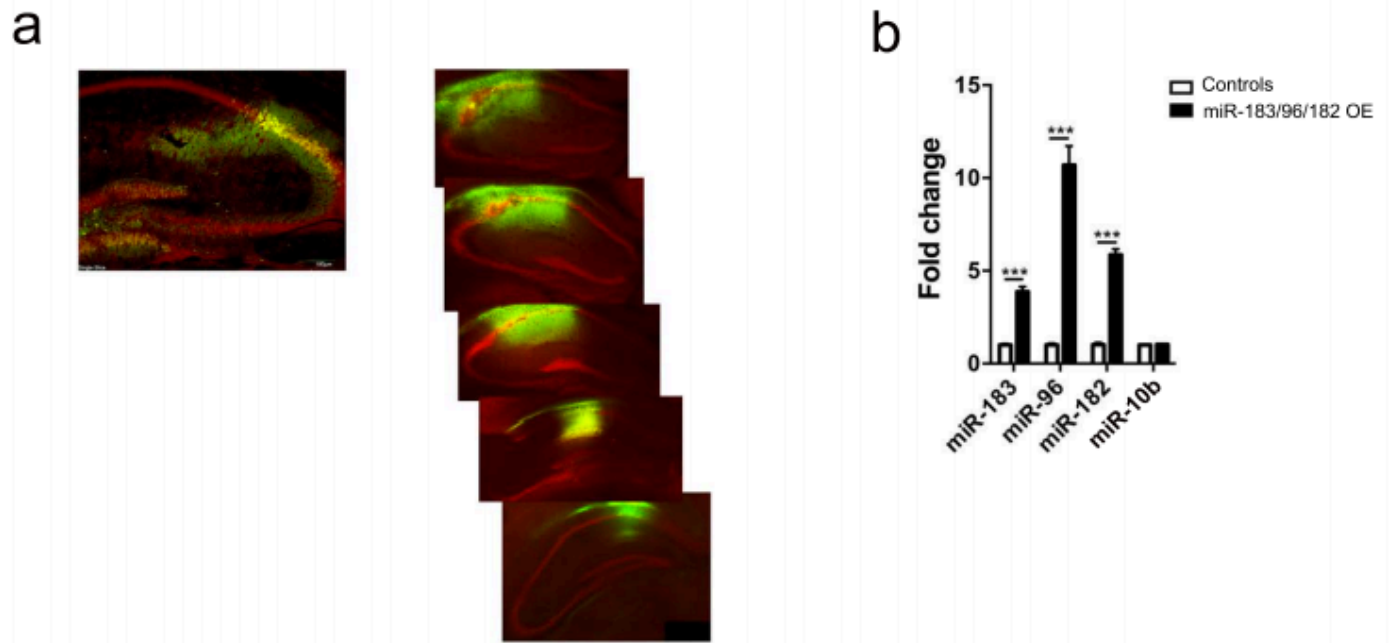
supplementary Figure 6: Effect of PP1 γ inhibition on miR-183/96/182 precursor and mature transcripts in N2A cells. (a) Representative Western blot of PP1 γ in whole cell extracts of N2A cells transfected with PP1 γ siRNA (left panel), and quantification of blots (right panel, $t_4=4.31$, $^*p<0.05$). (b) Mature miR-183/96/182 expression in N2A cells (whole cells fraction) after PP1 γ knockdown (miR-183: $t_4=3.26$, $^*p<0.05$; miR-96: $t_4=0.28$, $p=0.8$; miR-182: $t_4=2.33$, $\#p=0.08$). (c) Pri-miR-183/96/182 and pre-miR-183/96/182 expression in whole cell extract of N2A cells after 1hr KCl stimulation (pri-miR-183/96/182: $t_4=4.31$, $^*p<0.001$; pre-miR-183: $t_4=6.13$, $^{**}p<0.01$; pre-miR-96: $t_4=4.20$, $^*p<0.05$; pre-miR-182: $t_4=9.26$, $^{***}p<0.001$). (d) Cytoplasmic pre-miR-183/96/182 level in N2A cells after PP1 γ knockdown and 1hr KCl stimulation (pre-miR-183: $t_4=4.16$, $^*p<0.05$; pre-miR-96: $t_4=3.02$, $^*p<0.05$; pre-miR-182: $t_4=2.68$, $\#p=0.06$). (e) Level of mature ($t_4=1.29$, $p=0.27$) and precursor ($t_4=0.73$, $p=0.50$) transcripts of miR-10b in cells treated with PP1 γ siRNA. Bar graphs represent mean \pm s.e.m.



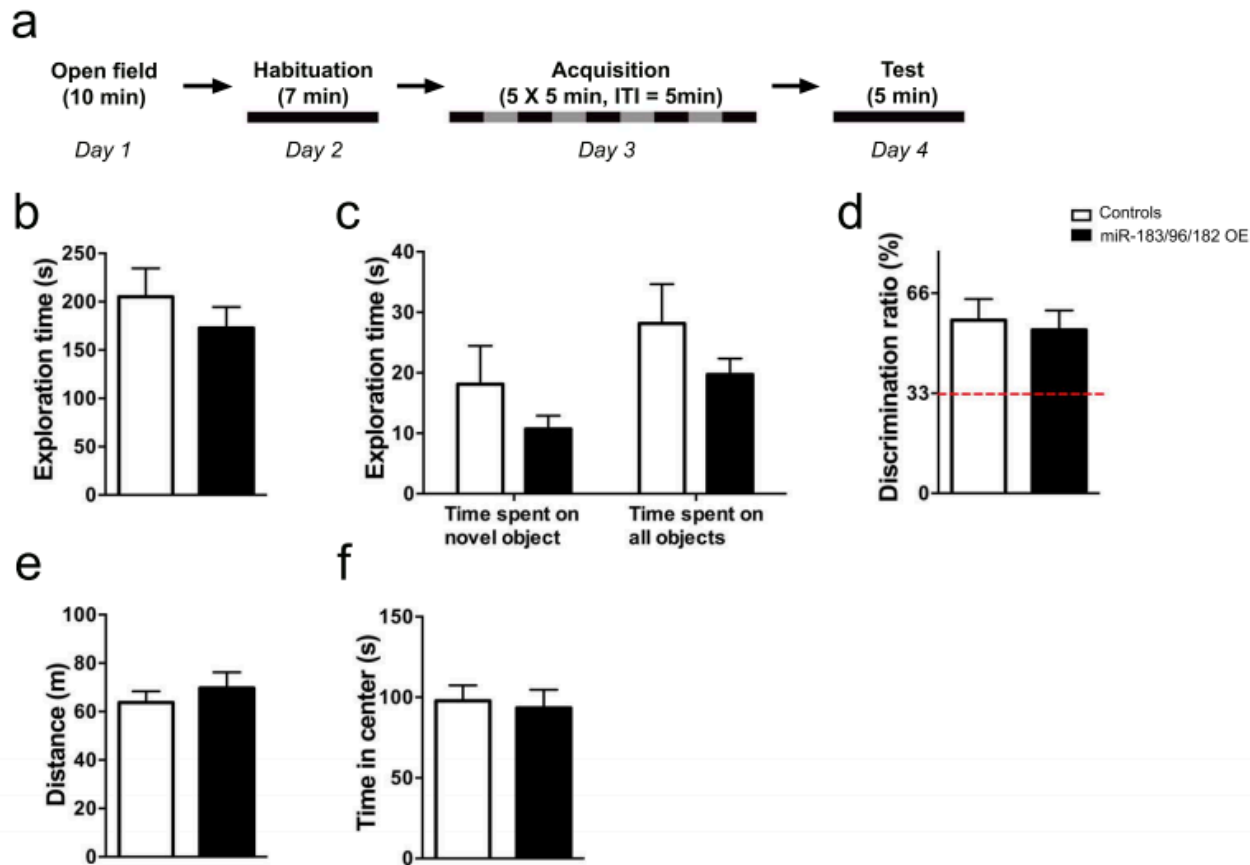
supplementary Figure 7: Verification of Actinomycin D treatment on c-Fos expression in N2A cells treated with KCl for 1hr; (Two-way ANOVA, ActD $F_{1,8}=29.77$, $p<0.001$. post-hoc: Control siRNA $t_8=3.86$, $^{**}p<0.01$; PP1 γ siRNA: $t_8=3.86$, $^{**}p<0.01$). Bar graphs represent mean \pm s.e.m.



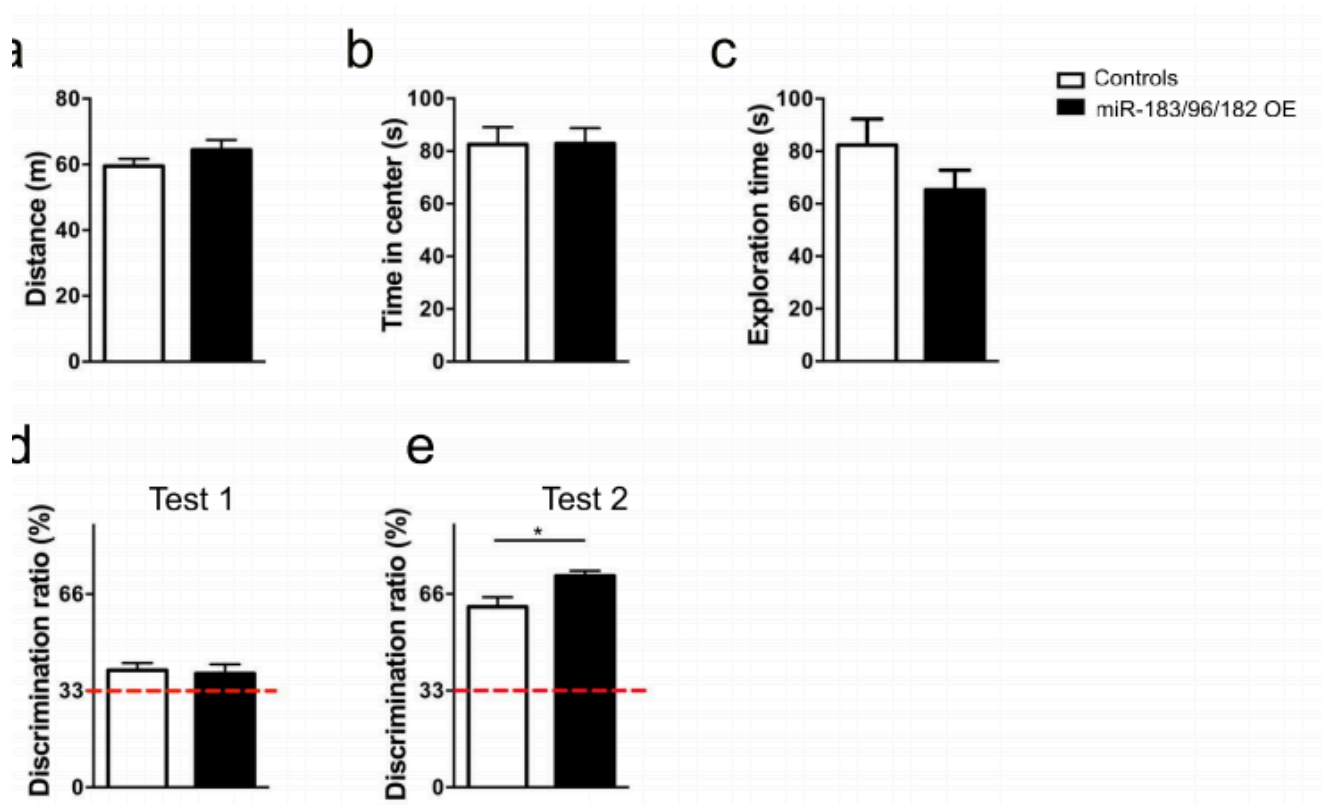
Supplementary Figure 8: MiRNA expression in hippocampus of mice trained with the strong NOR protocol and sacrificed at 1h, 3hr or 24hr after testing. (a) Left panel: pre-miR-183 one-way ANOVA $F_{3,23} = 1.156$, $p = 0.35$; right panel: pre-miR-182 one-way ANOVA $F_{3,23} = 4.00$, $p = 0.02$, posthoc (control, NOR 1hr) $**p < 0.01$. (b) Left panel: miR-183 one-way ANOVA $F_{3,23} = 0.79$, $p = 0.51$; right panel: miR-182 one-way ANOVA $F_{3,23} = 2.01$, $p = 0.14$. Bar graphs represent mean \pm s.e.m.



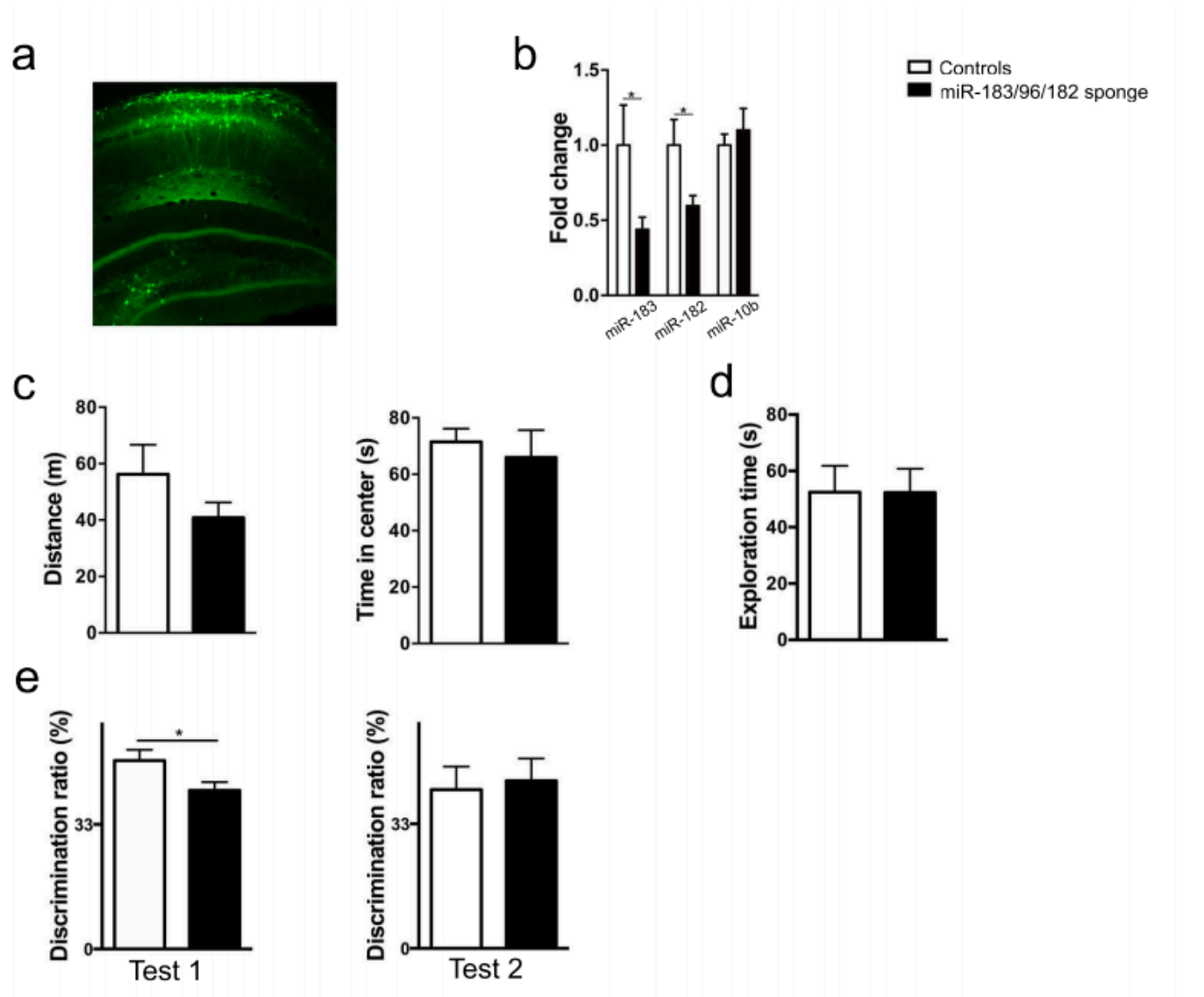
Supplementary Figure 9: AAV-mediated expression of miR-183/96/182 in the mouse hippocampus. (a) GFP staining of a brain slice 21 days after injection of a scAAV-cluster 183-GFP virus into the hippocampus. Neurons are labeled in red and GFP-expressing cells are green (anti-GFP). The series of images shown on the right panel display the extent of spread in the antero-posterior direction. (b) qPCR measurement of mature miR-183/96/182 levels in mouse hippocampus after injection with scAAV-control-GFP or scAAV-cluster 183-GFP (miR-183: $t_4=10.53$, *** $p<0.001$; miR-96, $t_4=9.43$, *** $p<0.001$; miR-182, $t_4=14.58$, *** $p<0.001$; miR-10b, $t_4=0.55$, $p=0.61$; controls, $n=3$; miR-183/96/182, $n=3$). Bar graphs represent mean \pm s.e.m.



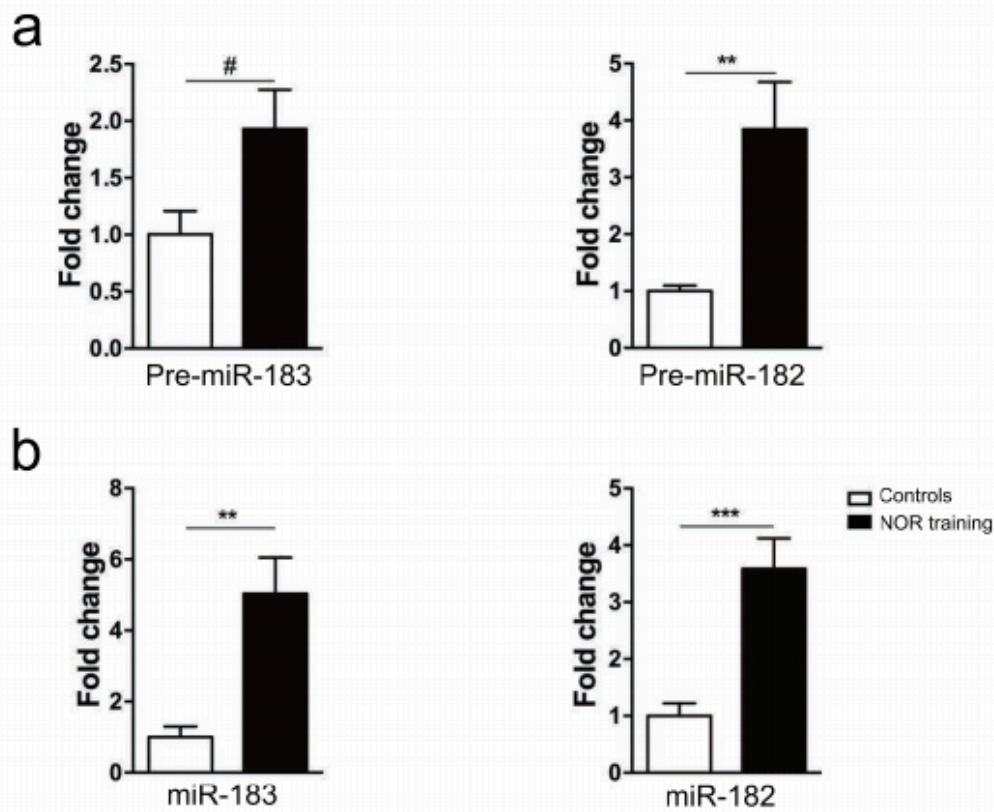
Supplementary Figure 10: MiR-183/96/182 overexpression (miR-183/96/182 OE) in the hippocampus does not influence long-term object memory with a strong training protocol. (a) Experimental setup. (b, c) Time spent exploring objects during acquisition (b, $t_{10}=0.88$, $p=0.40$), and testing on the NOR task (c, time on novel object: $t_{10}=1.1$, $p=0.29$; time on all objects: $t_{10}=1.2$, $p=0.26$) (d) Discrimination ratio of novel object over familiar objects (one-sample t-test, control: $t_5=3.43$, $p<0.05$; miR-183/96/182 - $t_5=3.27$, $p<0.05$; unpaired t test between controls and miR-183/96/182: $t_{10}=0.34$, $p=0.74$); the broken line shows chance level of discrimination set at 33%. (e, f) Total distance covered ($t_{10}=0.75$, $p=0.47$) (e), and total time spent in the center of the arena ($t_{10}=0.30$, $p=0.77$) (f), during open field-testing showing similar performance in control and miR-183/96/182 overexpressing mice. Controls, $n=6$; miR-183/96/182 OE, $n=6$. Bar graphs represent mean \pm s.e.m.



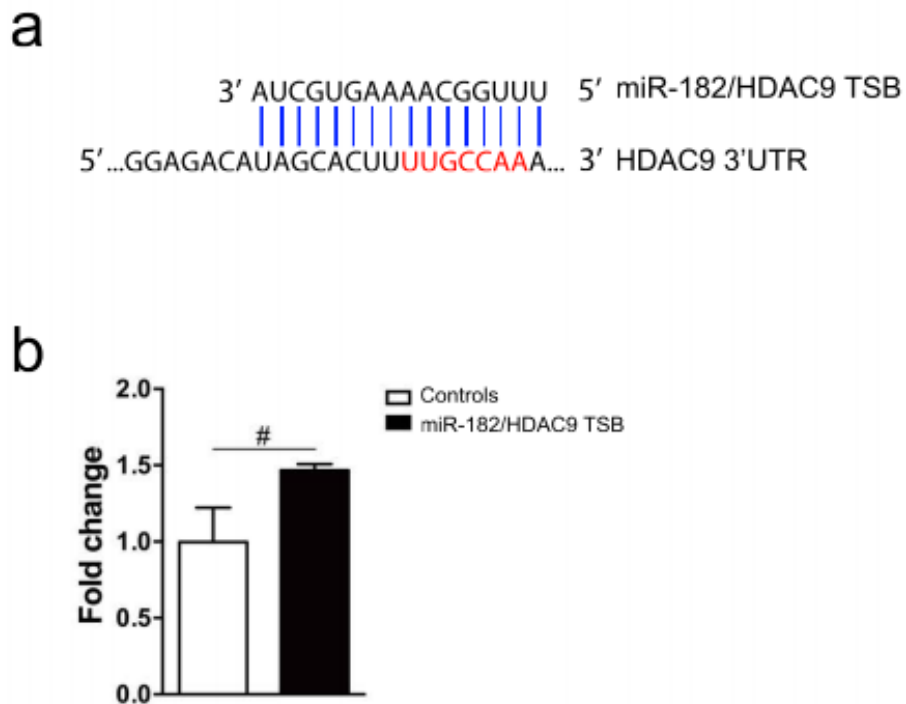
Supplementary Figure 11: Performance of miR-183/96/182 expressing (miR-183/96/182 OE) mice and controls in a weak training protocol. (a) Total path length during open field test ($t_{32}=1.30$, $p=0.20$); (b) Time spent in the center of the arena during open field test ($t_{32}=0.04$, $p=0.97$); (c) Time spent exploring objects during acquisition ($t_{32}=1.32$, $p=0.197$). (d) Discrimination ratio during test 1, 24hr after NOR training (One sample t-test, controls: $t_{18}=2.86$, $p<0.05$; miR-183/96/182, $t_{14}=1.84$, $p<0.1$; unpaired t-test between group comparison: ($t_{32}=0.3$, $p=0.77$). (e) Discrimination ratio during test 2, 48hr after NOR training (One sample t-test, control: $t_{18}=8.82$, $p<0.001$; miR-183/96/182: $t_{14}=22.37$, $p<0.001$; unpaired t-test between group comparison ($t_{32}=2.65$, $*p<0.05$). Controls, $n=19$; miR-183/96/182 OE, $n=15$. Bar graphs represent mean \pm s.e.m.



Supplementary Figure 12. Performance of mice expressing miR-183/96/182 sponge in the hippocampus under the weak NOR paradigm. (a) GFP expression in CA1 area of the hippocampus 10 days after scAAV-hSyn-miR-183 cluster sponge injection. (b) Quantification of miRNA expression in the hippocampus of mice expressing the sponge construct 30 days after virus injection (miR-183: $t_{14}=2.23$, $*p<0.05$; miR-182: $t_{14}=2.40$, $*p<0.05$; miR-10b: $t_{14}=0.57$, $p=0.58$); Control, $n=7$, miR-183/96/182, $n=9$. (c) Total distance covered (left panel: $t_{18}=1.31$, $p=0.21$) and time spent in center arena (right panel: $t_{18}=0.52$, $p=0.609$) during open-field test. (d) Total object exploration during 10min acquisition session ($t_{18}=0.01$, $p=0.99$). (e) Discrimination ratio 24hr (test 1: $t_{17}=0.25$, $*p<0.5$) and 48hr (test 2, $t_{16}=0.28$, $p=0.78$) after NOR training. Control, $n=9-10$; miR-183/96/182 sponge, $n=9-10$. Bar graphs represent mean \pm s.e.m.



Supplementary Figure 13: Hippocampal miRNA expression 30 minutes after training with the weak NOR protocol. (a) Left panel: pre-miR-183, $t_6=2.32$, # $p=0.06$; right panel: pre-miR-182, $t_7=3.01$, * $p=0.02$. (b) Left panel: miR-183, $t_9=4.15$, ** $p<0.01$; right panel: miR-182, $t_9=4.8$, *** $p<0.001$. Control, $n=4-6$; NOR, $n=5$. Bar graphs represent mean \pm s.e.m.



Supplementary Figure 14: miR-182/HDAC9 target site blocker (TSB) and its effect on HDAC9 expression. (a) Alignment of miR-182 TSB at HDAC9 3'UTR; sequences labeled in red are miR-182 seed sequence binding sites. (b) HDAC9 mRNA level in N2A cells treated with miR-182 TSB ($t_6=2.05$, $p=0.087$). Bar graphs represent mean \pm s.e.m.

Supplementary Table 1: List of primers, siRNAs, and plasmids

Primer/siRNA/plasmid	Source/sequence
miR-183, forward primer	Qiagen, cat. No.: MS00001722
miR-182, forward primer	Qiagen, cat. No.: MS00011291
miR-96, forward primer	Qiagen, cat. No.: MS00001456
miR-135a, forward primer	Qiagen, cat. No.: MS00011130
miR-135b, forward primer	Qiagen, cat. No.: MS00001575
miR-204-3p, forward primer	Qiagen, cat. No.: MS00024514
miR-146, forward primer	Qiagen, cat. No.: MS00001638
miR-200c, forward primer	Qiagen, cat. No.: MS00001827
Let-7c, forward primer	Qiagen, cat. No.: MS00005852
miR-190, forward primer	Qiagen, cat. No.: MS00032438
Pre-miR-10b, forward primer	Qiagen, cat. No.: MP00003983
miR-10b, forward primer	Qiagen, cat. No.: MS00032249
RNU6, forward primer	Qiagen, cat. No.: MS00033740
10X miScript Universal reverse primer	Qiagen, cat. No.: 218193
Pre-miR-96, forward & reverse primers	Qiagen, cat. No.: MP00006881
Pre-miR-183, forward & reverse primers	Qiagen, cat. No.: MP00004438
Pre-miR-182, forward & reverse primers	Qiagen, cat. No.: MP00004431
Pri-miR-183/96/182 forward	CCC TCC TAA AAC CAC CCT AA
Pri-miR-183/96/182 reverse	AGT TGG CAA GTC TAG AAC CAC
HDAC9, forward primer	ACG AGA AAG GGC AGT GGC AAG C
HDAC9, reverse primer	GAT GTG TGG TGG GCA GCC GT
NUFIP2, forward primer	AAC GCC GAA GAA GAA AAC AGG CTA
NUFIP2, reverse primer	GCT GAC ATC GGG ACC TGG GA
GAPDH, forward primer	CCA CTG GTG CTG CCA AGG CT
GAPDH, reverse primer	GGC AGG TTT CTC CAG GCG GC
Cacnb4, forward primer	TAC CTG CAT GGA GTT GAA GAC T
Cacnb4, reverse primer	TTC GCT CTC TCA AGC TGG ATA
Gabra1, forward primer	TGT GCG AGG GAG AGC AAG TC
Gabra1, reverse primer	AGC TAG GAA GCA GGG AGA TGT A
Prkcz, forward primer	GAC TGG GTG CAG ACA GAG AAA C
Prkcz, reverse primer	ACT CGA TGA CCA GGA ACA ACC G
Nrg1, forward primer	TGT GGT GGC CTA CTG CAA AA
Nrg1, reverse primer	TGG TGG GTT TGG ATG GTG AG
Ppp2ca, forward primer	ATG GAC GAG AAG TTG TTC ACC
Ppp2ca, reverse primer	CAG TGA CTG GAC ATC GAA CCT
Grm5, forward primer	CGT CTG GGG AAA CCC TAA GCT CCA
Grm5, reverse primer	TCA CCT CGA TGG CCG GCA GA
Gria1, forward primer	GTC CGC CCT GAG AAA TCC AG

Drosha, forward primer	GAG CCT AGA GGA AGC CAA ACA
Drosha, reverse primer	GCC GGA CGT GAG TGA AGA T
Dicer, forward primer	TTA ACC TTT TGG TGT TTG ATG AGT GT
Dicer, reverse primer	GCG AGG ACA TGA TGG ACA ATT
Ago2, forward primer	CCA TCT AGC TGT GAA GGC TCT GA
Ago2, reverse primer	TTC TTA GGG CCA GGC TTT AAA A
Gria1, reverse primer	CTC GCC CTT GTC GTA CCA C
Usp13, forward primer	CCC AGG GTA CAC GGG CCT GA
Usp13, reverse primer	GCT GAG GCT TGT GCT CCT CCT TC
Tubd1, forward primer	GGG AGA ATC ATG GAC CAG AA
Tubd1, reverse primer	TTG CTG CTG CTG TCT TTG TT
PP1 γ siRNA pool	ON-TARGETplus Mouse Ppp1cc (19047) siRNA- SMARTpool, 5nmol. ThermoScientific catalog number L-040212-00-0005
Negative control siRNA	AllStars Negative control siRNA (20 nmol). Qiagen, catalog Number 1027281
PP1 γ 3' UTR plasmid	Ppp1cc (GFP-tagged) - Mouse protein phosphatase 1, catalytic subunit, gamma isoform (cDNA clone MGC:13976 IMAGE:3487479), (10ug), 10 μ g. Origene, catalog number: MG204669

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3. Impaired PP1-dependent biogenesis of microRNA-183/96/182 underlies cognitive dysfunction associated with aging and TDP-43 proteinopathies

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3.1. Abstract

Molecular mechanisms underlying age-related cognitive decline (ARCD) remain largely unknown. We show that aging-induced increase in the nuclear activity of memory inhibitor protein phosphatase 1 (PP1) in the hippocampus neurons causes ARCD by impairing the microprocessor-mediated biogenesis of microRNA (miR) cluster, miR-183/96/182. Dysregulated PP1-dependent biogenesis of miR-183/96/182 may also contribute to the non-genomic blockade on cognitive functions in neurodegenerative disorders characterized by TDP-43 loss of function.

3.2. Main Body

Aging is associated with progressive deterioration of learning and memory formation termed as age-related cognitive decline (ARCD).¹ The molecular mechanisms underlying ARCD are largely unknown, but may involve protein phosphatase 1 (PP1) and Calcineurin (CaN), which are negative regulators of memory.^{2,3} We previously showed that PP1 regulates the nuclear biogenesis of memory-promoting microRNAs (miRs), miR-183/96/182, in a transcription-independent manner.⁴ Here, we show that increase in nuclear PP1 by aging selectively impairs the microprocessor processing of miR-183/96/182 by modulating the receptor SMAD (R-SMAD) dependent regulation of the microprocessor. We further show that a similar mechanism may contribute to the cognitive dysfunction in TDP-43 proteinopathies (CD TDP), and that artificial over-expression of miR-183/96/182 rescues ARCD.

Using a weak protocol of novel object recognition (NOR) training (**Supplementary Fig. 1**), we showed that aged (20-21 months old) mice perform worse than young (4-5 months old) mice when tested for their object recognition memory 24 and 48 hours post-training (**Fig. 1a**). We then quantified miR-183 and miR-182 in hippocampal tissue extracted from young and aged mice 30 minutes after NOR training. NOR training increased hippocampal miR-183 and 182 (**Fig. 1b**), as well as, pre-mir-182 and pri-mir-183-96-182 in young mice (**Fig. 1c**). However, aging prevented the NOR training-induced increase of hippocampal miR-183 and 182 at the precursor (**Fig. 1c**) and mature level (**Fig. 1b**). As PP1 regulates the post-transcriptional biogenesis of miR-183/96/182, we next investigated if suppression of miR-183/182 up regulation upon NOR training with aging was due to increased nuclear PP1. Indeed, hippocampal tissue from aged mice showed increased nuclear PP1 activity (**Fig. 1d**). This was accompanied by a decreased expression of PP1 inhibitory subunit PP1R1A, and increased expression of PP1 nuclear localization partner AKAP1, as well as, another phosphatase PPP2CA (**Supplementary Fig. 2**).

To show that microprocessor processing of pri-mir-183/96/182 is impaired by aging-induced increased in nuclear PP1, we conducted pri-mir-183 and 182 processing assays in mouse neuroblastoma (N2a) cells. This assays employs

renilla-luciferase dual-reporter system, in which cropping of the stem-loop sequence of the artificially introduced pri-mir-sequence reduces firefly signal, indicating microprocessor processing.^{4,5} Induction of senescence in N2a cells by hydroxyura treatment⁶ (**Supplementary Fig. 3**), as well as, over-expression of PP1 decreased the microprocessor processing of pri-mir-183/96 (**Supplementary Fig. 4**). To understand how PP1 regulates the microprocessor processing of pri-mir-183/96/182, we looked at the expression of major components of the microprocessor complex after PP1 manipulation in N2a cells. Over-expression of PP1 or its nuclear inhibitor (NIPP1) in N2a cells did not alter nuclear protein expression of Drosha/DGCR8 (**Supplementary Fig. 5**). Further, we could not detect a direct interaction of PP1 with the microprocessor in Drosha co-IP experiments (**Supplementary Fig. 6: data not shown**), suggesting involvement of an intermediate. R-SMAD proteins are down-stream signal transducers of transforming growth factor-beta (TGF-beta) and bone-morphogenic protein (BMP) that regulate the biogenesis of certain microRNAs by interacting with the SMAD binding element (SBE) on their primary transcripts, as well as, the microprocessor component p68.^{7,8} Over-expression of NIPP1 in N2a cells increases the phosphorylation of SMAD1/5 at Serine 463/465 required for their nuclear localization and interaction with p-68 (**Supplementary Fig. 7**).⁷ These results suggest that aging-induced increase in nuclear PP1 can decrease the microprocessor processing of miR-183/96/182. Indeed, hippocampal expression of miR-21, previously shown to be regulated post-transcriptionally by R-SMADs is altered by PP1 manipulation in N2a cells (**Supplementary Fig. 8**). On the other hand, microprocessor processing of pri-mir-183, but not of a control miRNA (pri-mir-10b), showed dependence on R-SMAD signaling (**Supplementary Fig. 9**).

We next attempted to reverse ARCD in aged mice. For this, we overexpressed miR-183/96/182 in hippocampus area CA1 using a self-complementary adeno-associated virus (scAAV) vector expressing pre-miR-183/96/182 fused with GFP. Virus transduction and miRNA overexpression were confirmed by immunohistochemistry and qPCR (**Supplementary Fig. 10 data not shown**). Importantly, aged mice with hippocampal over-expression of miR-183/96/182 performed comparable to young mice when tested for their object recognition memory at both 24 and 48 hours after NOR training (**Fig. 1e**). This result

reiterates the previously shown role of miR-183/96/182 in long-term memory, and highlights their potential to correct ARCD.⁴ To further validate the implication of PP1-miR-183/96/182 pathway in ARCD, we measured nuclear PP1 activity in hippocampal tissue from aged mice, which were exposed to environment enrichment (EE) for four weeks (**Supplementary Fig. 11: data not shown**). EE, which is known to rescue ARCD,^{1,9} decreased nuclear PP1 activity (**Fig. 1f**) by increasing the expression of PP1 inhibitory subunits PP1R1A and NIPP1 (**Fig. 1g**). Further, decrease in PP1 nuclear activity with EE also lead to an increase in miR-183/182, as well as, their precursors (**Supplementary Fig. 12**).

Emerging evidence suggests that changes in miRNAs expression and functioning impair memory in neurodegenerative disorders.¹⁰ miRNA-mediated pathways may arbitrate cognitive dysfunction in these disorders both by promoting neuronal death and through changes in memory relevant gene expression, especially in cases of early and subtle impairments in memory preceding neuronal death.¹⁰⁻¹² Furthermore, it has been recently shown that increased activity of histone deacetylases (HDACs) induces an epigenetic blockade on memory formation in the neurodegenerating brain.¹³ To check if increase in nuclear PP1 and resulting decrease in biogenesis of miR-183/96/182, which target HDAC9,⁴ could contribute to such non-genomic blockade of memory, we performed pri-mir-183/182 assays under different loss of nuclear function conditions simulating neurodegenerative disorders *in vitro*; α -synuclein knock down (Parkinson disease), and TDP-43/ FUS knock-downs (amyotrophic lateral sclerosis/ frontotemporal lobar degeneration) (**Supplementary Fig. 13: data not shown**). TDP-43 knock down impaired microprocessor processing for both pri-mir-183 and pri-mir-182 (**Fig. 2a**) that could be rescued in case of pri-mir-183 by pharmacological inhibition of PP1 (**Supplementary Fig. 14**), suggesting that the decrease in microprocessor processing of miR-183/96/182 on TDP-43 loss of function involves PP1. Indeed, knock down of TDP-43 lead to an increase in nuclear PP1 activity in N2a cells (**Fig. 2b**) without changing the expression of Drosha or DGCR8 (**Supplementary Fig. 15**). We further analyzed the expression of miR-183/96/182 in frontal cortex tissue from patients with amyotrophic lateral sclerosis (ALS), a neurodegenerative condition characterized by TDP-43

pathology (**Supplementary Fig. 16: data not shown**) and cognitive dysfunction in almost 50% of the patients.^{14,15} miR-183/96/182 and their precursors (**Supplementary Fig. 17: data not shown**) were decreased in ALS (**Fig. 2c**), with a corresponding increase in the expression of their targets (**Fig. 2d**), and PP1 γ , the predominantly nuclear isoform of PP1 (**Fig. 2e**).

Taken together, our findings identify impaired microprocessor processing of miR-183/96/182 resulting from increased nuclear PP1 as an important pathogenic mechanism underlying ARCD and CD TDP. This memory impairment pathway involves R-SMADs as potential intermediates, and is amenable to therapies targeted at decreasing neuronal nuclear PP1 and/or artificially increasing miR-183/96/182 in the hippocampus (**Fig. 2f**).

3.3. Materials & Methods

Animals

Young (3-4 months old) and aged (>18 months old) C57Bl/6 mice were purchased from Janvier (France). The mice were group-housed (4 mice per cage) under a reverse 12h light/dark cycle (25°C, 55% humidity), with food and water *ad libitum*. Behavioral experiments were carried during the animals' dark cycle. Experiments and animal maintenance were conducted in compliance with the Federation of Swiss Cantonal Veterinary Office and approved by Zürich Cantonal Veterinary Office (54/2012).

Novel object recognition (NOR) task

Object recognition training was conducted in a rectangular arena (60cmx50cmx45cm), with gray, opaque walls and translucent plexiglass bottom, under which an infrared light source was placed. It was located in a dedicated behavioral room illuminated by a dim light. Before NOR training, each animal was handled daily for 4 minutes on 4 consecutive days. This was followed by an open field test, which involved placing each mouse in the empty arena for 10 minutes and measuring their overall locomotion activity. An additional habituation to the empty arena was conducted one day before the NOR training. For training (acquisition), three different unfamiliar objects were placed in the center of the arena in a triangular arrangement. Each animal was allowed to explore the objects for a single 10-min session. Object memory was

tested in 5-min sessions for which one of the familiar objects was replaced with a novel object at 24h (test1) and 48h (test2) after training. The time that an animal spent exploring each object during testing was measured with a video tracking system (ViewPoint Behavior Technology) by an experimenter blinded to group assignment. Object memory was expressed as the proportion of time spent exploring the novel object compared to the time spent exploring all objects (discrimination index). The discrimination index was normalized taking the average value from control animals as 100%. The animal movements were tracked by an infrared camera connected to a tracking software (View Point Behavior Technology). For miRNA expression experiments, NOR controls (identified as 'habituation') were subjected to identical procedures without an exposure to objects.

Environment enrichment (EE)

Aged (19-21 months) and young adult (4-5 months) C57Bl/6J male mice were either housed in standard cages or in environmentally enriched cages for 4 weeks. Standard housing consisted of a clear polycarbonate standard Aero cages (391 x 199 x 160 mm) containing wood chip bedding and nesting material. Enriched housing consisted of a clear polycarbonate type 2000P cage (610 x 435 x 215 mm) containing wood chip bedding, nesting material, two running wheels, a climbing ladder, a jungle gym, a plumbing pipe, multiple plastic balls, a rubber ball and a wooden stick.

Cell culture

Mouse neuroblastoma (N2A) cells were obtained from American Type Cell Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle's Medium (DMEM- high glucose), supplemented with 10%(v/v) FBS (Gibco®) and 1% Antibiotic-Antimycotic (Gibco®). These cells were chosen for their fast-growth, easy maintenance and transfection, and potential for neuronal differentiation making them a convenient model for studying miRNA biogenesis. The cells were proven free of mycoplasma contamination through regular tests. Before the start of experiments, the cells were passaged 1:4 and split every 3 days for at least 5 passages. On transfection day, 150,000 - 300,000 cells were plated in 6 or 12-well plates. Transfection of a pool of siRNAs targeting TDP-43/FUS/ α -synuclein (Flexitube siRNA, Qiagen) or negative control siRNA (All

Star negative control, Qiagen) was carried out with HiPerfect[®] transfection reagent (Qiagen). Inhibition and over-expression of nuclear PP1 was achieved by overexpression of plasmids containing NIPP1 and PP1 γ open read frame constructs respectively (Origene). Then the cells were returned to the incubator and allowed to grow for 48 hours prior to harvest or further treatment. The cells were harvested by removing the medium, washing with ice-cold PBS three times, and lysing with Tri-reagent[®] (for RNA extraction) or RIPA buffer (for protein extraction) or 0.05% Trypsin-EDTA (for sub-cellular fractionation). All experiments were conducted on at least three replicates from different passage number, and repeated at least three times.

Drug Treatments

Cellular senescence was induced by treating N2a cells with 8 or 16mM hydroxyurea (HU, Sigma-Aldrich, St. Louis, MO, USA) for an incubation time (37°C, 5% CO₂) of 12 h. HU treatment was done 12 h prior to cell lysis. H₂O was used as a control. Induction of cellular senescence was confirmed by checking the expression of senescence markers p21 and p53. Primer sequences: p21 (Fwd: TACTTCCTCTGCCCTGCTGC, Rev: GCTGGTCTGCCTCCGTTTT), p53 (Fwd: CACGTACTCTCCTCCCCTCAAT, Rev: AACTGCACAGGGCACGTCTT). Protein phosphatases were inhibited by treating N2a cells with Okadaic acid (Cell Signaling) at two different doses; 20 nM (inhibition of PP1/PP2A), and 0.1 nM (inhibition of PP2A only).¹⁶

Nuclear PP1 activity assay

Nuclear PP1 activity assay was performed as previously described.¹⁷ Briefly; hippocampi were dissected and homogenized in 500 ul of cytoplasmic extraction buffer (Subcellular Protein Fractionation Kit for Tissue, Thermo Fischer scientific), transferred to Pierce Tissue Strainer and centrifuged at 500g for 5 minute. The supernatant containing the cytoplasmic protein fraction was isolated, and the pellet containing the nuclear fraction was incubated on ice for 10 minutes, and centrifuged at 3000g for 5 minues in 325 ul membrane extraction buffer. The pellet containing the nuclei was resuspended in 110 ul of nuclear extraction buffer (NEB), incubated for 30 minutes, and centrifuged at 5000g for 5 minutes. The supernatant containing soluble nuclear extracts was desalted using PiBind resin (Innova Biosciences). Phosphatase activity was

determined by incubating 20 μ l of the isolated nuclear fraction with 0.75 mM RII phosphopeptide substrate (BIOMOL), with or without 5nM Tautomycin (selective inhibitor of PP1 at 5 nM, ENZO Life Sciences) at 30°C for 10 minutes. The amount of free phosphate released was measured with BIOMOL green reagent (BIOMOL) at 620 nm and background subtracted. Nuclear PP1 activity was calculated by subtracting the nuclear phosphatase activity with Tautomycin from the nuclear phosphatase activity without Tautomycin.

Luciferase-based pri-miRNA processing

Pri-miRNA processing assays were conducted as previously described.^{4,5} Briefly, fragments of pri-mir-182 and pri-mir-183 containing the hairpin and 100 bp flanking sequence were amplified from genomic DNA. The PCR products were digested with the respective restriction enzymes and inserted at MCS in pmirGLO vector (Dual Reporter Luciferase Assay System, Promega) downstream to firefly luciferase reporter. Cropping of the hairpin stem-loop of the inserts results in destabilization of the firefly reporter leading to a decrease in firefly luminescence. The unperturbed renilla reporter produces stable luminescence, which serves as internal normalization control. Dual-luciferase reporters with pri-mir-182, and pri-mir-183 were transfected in N2a cells using cationic liposomes (Lipofectamine 2000 reagent, Invitrogen). The cells were lysed 48 hr post-transfection with passive lysis buffer (Promega) treatment at room temperature for 10 minutes. The lysates were then transferred to a 96 well plate, and luciferase activities of firefly and renilla were read through luminometer GloMax 96 (Promega) equipped with dual injections dispersing LAR II (for firefly luciferase quantification) and Glomax (for renilla luciferase quantification) reagents sequentially.

Production of plasmid for miRNA overexpression

The scAAV2-EF1a-pri-miR-183/96/182-GFP construct was produced by cutting scAAV2-MCS (Cell Biolabs) by Ball/NotI (New England Biolabs). The transgene cassette containing Ef1a promoter (sequence from pEGP-mmu-miR-182 plasmid; Cell Biolabs), engineered truncated (T)-pri-miR-183/96/182, EGFP (from pEGP-mmu-miR-182 plasmid; Cell Biolabs), WPRE motif and 5'-Ball and 3'-NotI adapters was chemically synthesized by GENEWIZ (South Plainfield, USA) and cloned into the scAAV backbone. Sequence of T-pri-miR-

183/96/182 was as follows with mature miRNAs underlined: 5'cctctgcaggggtctgcagggctggagagtgtagctcctgtcctgtgtatggcactggtagaatt cactgtgaacagtctcagtcagtgattaccgaagggccataaacagagcagagacagatccgcgagca ccttgagctcctcaccctttctgcctagacctctgtttccaggggtgccaggggtacaaagacctcctctgctc ctccccagagggcctgtccagtaccatctgcttggccgattttggcactagcacattttgcttgtgtctctccgct gtgagcaatcatgtgtagtgccaatatgggaaaagcgggctgctgcggccacgttcacctccccggcatcc cataataaaaacaagtatgctggaggcctcccaccattttggcaatggtagaactcacaccggtaaggtaat gggacccggtggttagacttgccaactatggtgtaagtgtgagct. The scAAV2-EF1a-pri-miR-183/96/182-GFP allowed the generation of mature miR-183-5p, miR-96-5p, and miR-182-5p sequences annotated in miRBASE v.20 (www.mirbase.org), whose expression was verified both by RT-qPCR and Northern blot analysis in HEK293 cells transfected with scAAV2-EF1a-pri-miR-183/96/182-GFP plasmid (data not shown). We also verified that expression of these miRNAs in HEK293 cells leads to specific repression of reporter mRNAs bearing miR-183/96/182 sites (data not shown). Control scAAV2-EF1a-control-GFP construct contained fragment of beta-globin intron (sequence from pEGP-mmu-miR-182 plasmid; Cell Biolabs) of length corresponding to T-pri-miR-183/96/182.

scAAV production

Self-complementary AAV production was performed by triple transfection of HEK 293T cells using polyethylenimine with a plasmid bearing the target sequences between the internal terminal repeats of scAAV2, the AAV-helper plasmid encoding Rep2 and Cap for serotype 8, and the pHGTI-Adeno1 plasmid harboring helper adenoviral genes (both kindly provided by C Cepko, Harvard Medical School, Boston, MA, USA). Vectors were purified using a discontinuous iodixanol gradient (Sigma, Optiprep). Encapsidated DNA was quantified by TaqMan RT-PCR following denaturation of the AAV particles by Proteinase K, and titers were calculated as genome copies (GC) per ml.

Stereotaxic surgery and intra-hippocampal injections

To overexpress miRNAs, virus vectors were prepared as described above. The oligonucleotides were stereotaxically injected in to the CA1 region of the hippocampus at a concentration of 1ug/ul, in TurboFect in vivo transfection reagent (Dharmacon). To carry out injections, animals were anaesthetized with

3% isoflurane (Attane™) and placed in a stereotaxic frame. Anaesthesia was maintained with 1.5% isoflurane in 100% oxygen throughout the surgery. Injection was carried out by lowering a glass pipette (Blaubrand®, cut to a 20µm inner diameter) filled with virus/oligonucleotides and attached to an injection pump (Stoelting) through a predrilled hole at the following coordinate targeting CA1 region of the hippocampus (from Bregma): *AP*: -2.0mm, *ML*: +/- 1.5mm, *DV*: -1.6mm. A total of 1µl (10⁹GC/ml) of virus vector or 1.5µl of TSB oligos was injected into each hippocampus at a rate of 0.2µl/min. The glass pipette was left in place for an additional 5 minutes, before it was carefully withdrawn and the wound was closed. The animals were allowed to recover for up to 2 weeks in their home cage before behavioral testing.

RNA extraction and RT-qPCR

Mouse hippocampal tissue was homogenized using TissueLyser (Qiagen) in Trizol® reagent (Invitrogen). Total RNA was extracted by phenol-chloroform precipitation. For extraction from cells, the medium was removed, the cells were washed three times with ice cold-PBS, lysed and homogenized by adding Trizol® directly to the plates. Subcellular fractionation of nuclear and cytoplasmic RNA was performed using Norgen's Cytoplasmic and nuclear RNA purification kit (Norge BioTek, Canada). One microgram of total RNA was treated with RNase free DNase (Promega) and reverse transcribed using miScript II RT kit® (Qiagen). MiScript primer assays for mature and precursor miRNAs were used to amplify the respective transcripts from a cDNA pool (Supplementary Table 1). For mRNA quantification, custom designed or Quantitect (Qiagen) gene specific primers were used (Supplementary Table: under preparation). Real time PCR was performed on LightCycler 480® (Roche). Small nuclear RNA (RNU6) or GAPDH was used as endogenous control and quantification was performed as previously.¹⁸

Protein extraction and Western blot

Total protein was extracted from N2A cells using radio immunoprecipitation buffer (RIPA) with 1:1000 protease inhibitor cocktail (Sigma-Aldrich) and 1:500 phenyl methyl sulfonyl fluoride (PMSF). Cells were lysed directly on the culture plate with 100-150 µl RIPA and scraped off with a cell scraper. The lysate was transferred to a micro centrifuge tube and sonicated for 5 cycles, each

comprising 30 seconds of sonication with 30 seconds intervals. The resulting mixture was centrifuged for 15 minutes at 14,000g to separate the protein mixture (supernatant) from cellular debris (pellet). Nuclear proteins were extracted from N2a cells as described in the section 'PP1 activity assays'. 20-40 ug proteins were resolved on SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-rad). Membranes were blocked in 3% BSA for one hour, and then incubated in primary (overnight at 4°C) and secondary (one hour at room temperature) antibodies. They were scanned using Odessey IR scanner (Li-Cor Bioscience), and band intensity was determined and quantified using image analysis software (ImageJ). The following antibodies were used: primary – phospho SMAD 1/5 Ser 463/465 (Cell Signaling Technology, mAb 9516), Drosha (Abcam, ab135956), DGCR8 (Abcam, ab35865), Cyclophilin A (Abcam, ab58144), HistoneH3 (Abcam, ab 1791), GAPDH (Abcam, ab 9485); secondary – anti-mouse IRDye® goat anti-mouse (LI-COR, 925-32210) and IRDye® goat anti-rabbit (LI-COR, 925-32211).

Statistical analysis

To confirm discrimination of novel object by the animals during memory testing against a predefined chance level, one-sample Student t test was used. For behavioral/molecular data comparison between two groups, unpaired Student t test was used, with additional Welsch correction in cases where the variances were unequal between the two groups. For data involving comparison between more than two groups, one-way ANOVA was used. To check the effect of aging on miRNA upregulation upon learning, two-way ANOVA was employed. Significant ANOVA analyses were followed by post-hoc pair-wise comparisons. Tukey's post-hoc was used when pair-wise comparisons between all the groups were required. In selected cases requiring pair-wise comparisons between a specific group versus the control, Sidak post-hoc was used. Outliers were defined as values beyond two standard deviations from the group mean and were removed from the analysis. Significance was set at $p < 0.05$ for all tests and two-sided tests were performed. Statistical analysis was performed using GraphPad prism version 7 and verified by SPSS version 23. All graphs were drawn with GraphPad prism version 7.

3.4. Acknowledgments

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3.5. Authors' contribution

A.J and I.M.M initiated and designed the study, analyzed the results and wrote the manuscript. A.J performed behavioral experiments, did molecular analysis of brain samples, and designed and executed *in vitro* experiments. B.T.W performed stereotaxic injections, co-immunoprecipitation experiments with N.G., and helped in the interpretation and drafting of *in vivo* data. E.A.K. conducted environment enrichment and performed luciferase assays with A.J. N.G and A.J conducted qPCR experiments and Western blots. M.P. and J.R. provided human samples with TDP-43 pathology and critical input to the manuscript.

3.6. Figures and Figure Legends

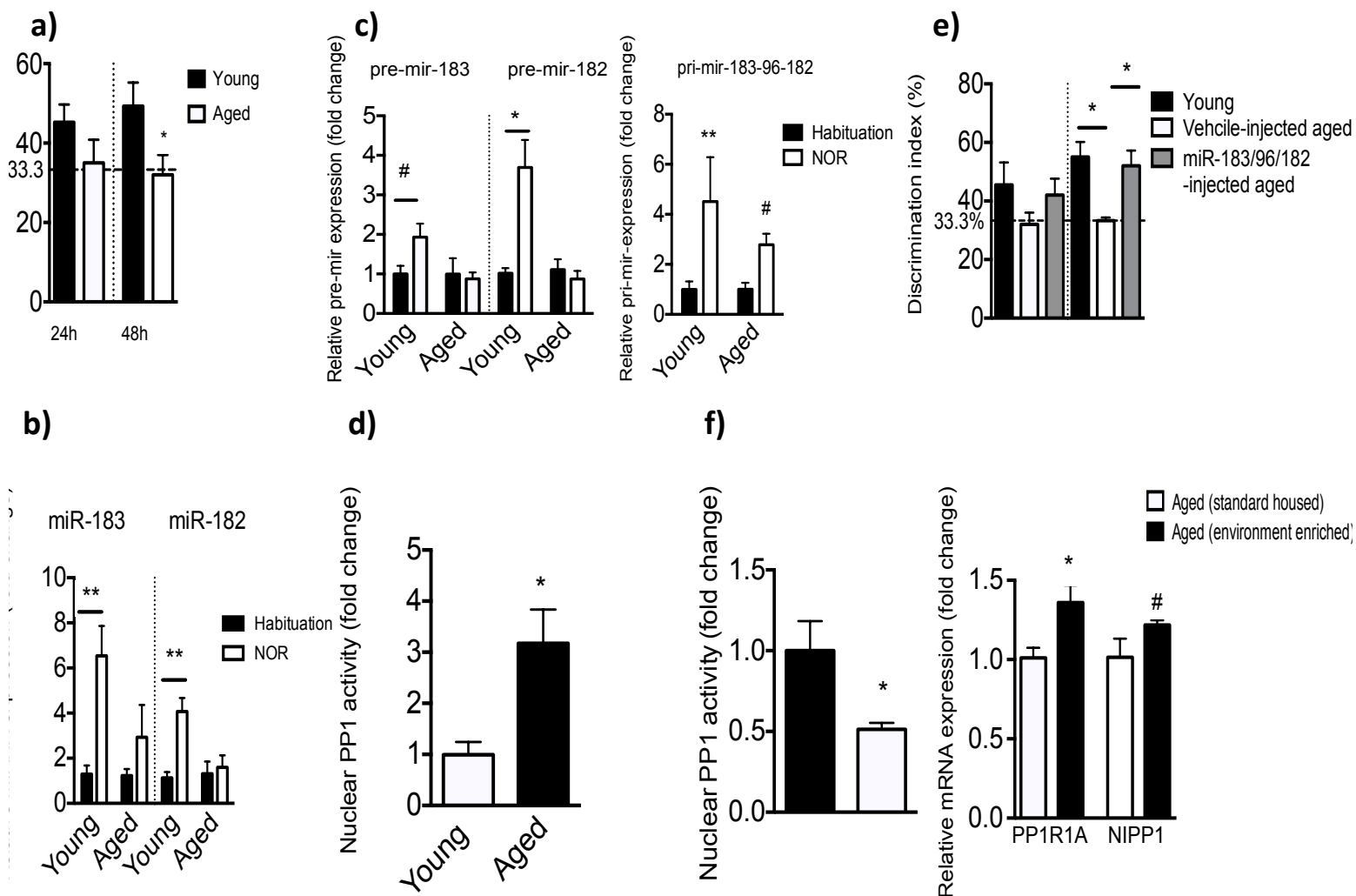


Fig. 1. miR-183/182 and their regulation by nuclear PP1 are implicated in ARCD.

(a) Performance of young (4-5 months old) and aged (20-21 months old) mice tested 24 and 48 hours after NOR training, expressed as discrimination index. At both 24 and 48 hours, only young animals demonstrate significant discrimination for the novel object (chance level set at 33.3%) (24 hours: one-sample t-test, control: 33.3%, $p<0.05$; 48 hours: one-sample t-test, control: 33.3%, $p<0.05$). Additionally, young mice had better performance than aged at 48 hours (unpaired t test: * $p<0.05$). Young, $n=9$, aged, $n=8$.

(b). Young (4-5 months old) and aged (20-21 months old) mice were trained on an NOR task. Hippocampal samples were collected from each group 30min after the end of training. The effect of NOR training on hippocampal expression of miR-183 and 182

was compared between young and aged mice. NOR training increased miR-183 (left) and 182 (right) in young, but not in aged mice (two-way ANOVA: miR-183: $F_{1,17}=3.399$, $p=0.0021$, post-hoc: young $**p<0.01$, aged $p=0.618$, miR-182: $F_{1,17}=11.04$, $p=0.004$, post-hoc: young $**p<0.01$, aged $p=0.978$). Young, $n=6$, aged, $n=6$.

(c) NOR training increased pre-mir-182 (left panel) in young (4-5 months old), but not in aged (20-21 months old) mice. However, NOR training increased pri-mir-183/96/182 in both young and aged mice (right panel) (two-way ANOVA: pre-mir-183: $F_{1,15}=1.555$, $p=0.23$, post-hoc: young $p=0.15$, aged $p=0.99$; pre-mir-182: $F_{1,15}=5.908$, $p=0.028$, post-hoc: young $*p<0.05$, aged $p=0.99$; pri-mir-183/96/182: $F_{1,15}=22.42$, $p=0.0006$, post-hoc: young $**p<0.01$, aged $\# p<0.1$). Young, $n=6$, aged, $n=6$.

(d) Increased nuclear PP1 activity in hippocampal tissue collected from aged (20-21 months old) mice compared to young (<6 months old) (unpaired t test: $*p<0.05$). Aged, $n=4$, young, $n=4$.

(e) Hippocampal over-expression of miR-183/182 in aged mice reverses age-related memory decline. Memory performance of young (4-5 months old), vehicle-injected aged (20-21 months old), and miRNA-injected aged (20-21 months old) mice was tested 24 and 48h after NOR training, expressed as discrimination index. Young mice demonstrate significant discrimination of the novel object at both 24 and 48h (24 hours: one-sample t-test, control: 33.3%, $p<0.1$; 48 hours: one-sample t-test, control: 33.3%, $p<0.05$). miRNA-injected aged mice show significant discrimination of the novel object at 48h (one-sample t test, control: 33.3%, $p<0.01$). MiRNA-injected aged mice show increased discrimination for the novel object as compared to vehicle-inject aged mice at 48h (one-way ANOVA, $F_{2,14}=6.018$, $p=0.01$, post-hoc: young $*p<0.05$, miR-183/96/182 injected aged $*p<0.05$). Young, $n=4$, vehicle-injected aged, $n=8$, miR-183/96/182 injected aged, $n=8$.

(f) Environment enrichment (EE) decreased hippocampal nuclear PP1 activity in aged mice. Left panel: nuclear PP1 activity in the hippocampal tissue collected from aged (19-21 months old) mice exposed to four weeks of EE was significantly decreased compare to standard-housed aged (19-21 months old) mice (unpaired t test, $*p<0.05$). Right panel: Increased mRNA expression of PP1 inhibitor PP1R1A and NIPP1 in hippocampal tissue collected from aged (19-21 months old) mice exposed to four weeks of EE compared to standard-house aged (19-21 months old) mice (unpaired t test: PP1R1A: $*p<0.05$, NIPP1: $\#p<0.1$). Standard housed aged, $n=8$, aged with EE, $n=8$.

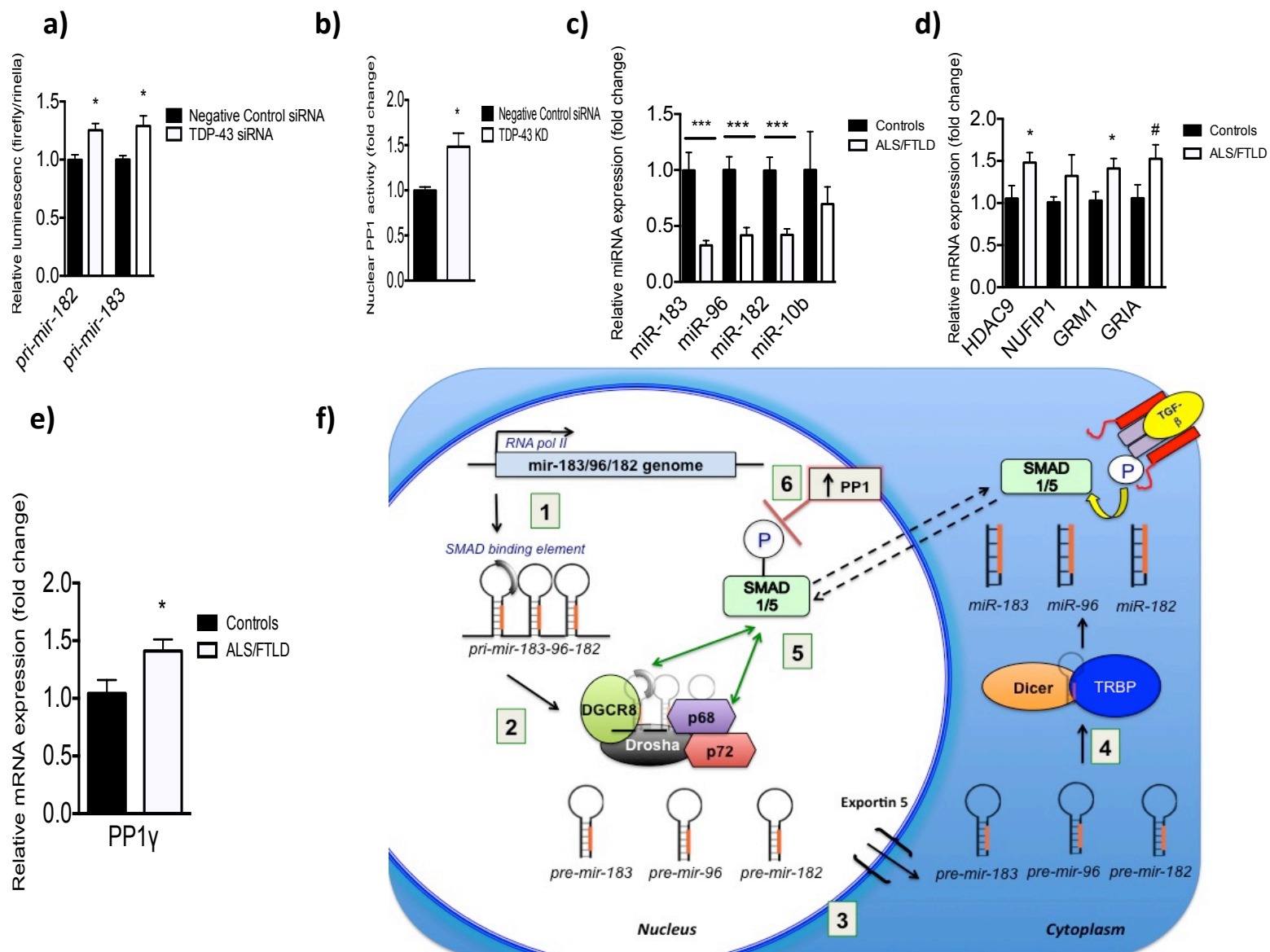


Fig.2. miR-183/182 and their regulation by nuclear PP1 contribute to non-genomic blockade of cognitive functions in TDP-43 proteinopathies.

(a) Decreased microprocessor processing of pri-mir-183 and pri-mir-182 in mouse N2a cells with TDP-43 knock-down. N2a cells were transfected with dual-luciferase reporters harboring pri-mir-183 and pri-mir-182 under TDP-43 (right) siRNA-mediated knock downs. TDP-43 knock-down decreased microprocessor processing of pri-mir-183 and pri-mir-182 (right), expressed as increased firefly/renilla luminescence (unpaired t test: pri-mir-183: * $p < 0.05$; pri-mir-182: * $p < 0.05$). Negative control siRNA, $n = 4$, α -synuclein siRNA, $n = 4$, FUS siRNA, $n = 4$, TDP-43 siRNA, $n = 4$.

(b) Increased nuclear PP1 activity in mouse N2a cells treated with TDP-43 siRNA compared to Negative control siRNA (unpaired t test, * $p < 0.05$). Negative control siRNA, $n = 4$, TDP-43 siRNA, $n = 4$.

(c) Decreased expression of miR-183, miR-96, and miR-182, but not of a control miRNA miR-10b compared to age-matched controls in cortical tissue collected from

patients with ALS/FTLD (unpaired t test: miR-183: *** $p < 0.001$; miR-96: *** $p < 0.001$; miR-182: *** $p < 0.001$; miR-10b $p = 0.389$). Controls, $n = 8$, ALS/FTLD, $n = 10$.

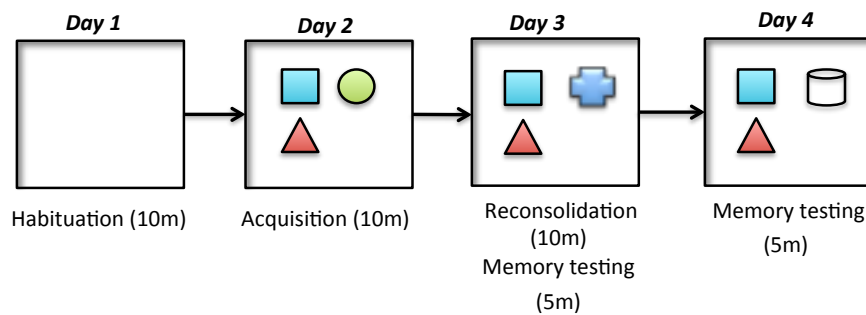
(d) Increased expression of targets of miR-183/96/182 compared to age-matched controls in cortical tissue collected from patients with ALS/FTLD (unpaired t test: HDAC9: * $p < 0.05$; NUFIP1: $p = 0.276$; GRM: * $p < 0.05$; GRIA: # $p < 0.1$). Controls, $n = 8$, ALS/FTLD, $n = 10$.

(e) Increased expression of PP1 γ compared to age-matched controls in cortical tissue collected from patients with ALS/FTLD (unpaired t test, * $p < 0.05$). Controls, $n = 8$, ALS/FTLD, $n = 10$.

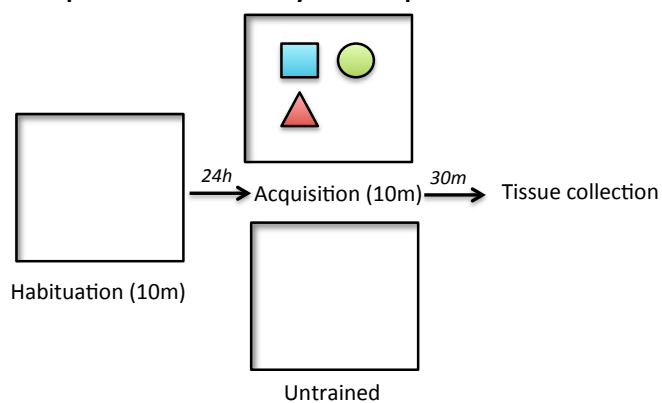
(f) Model representing regulation of miR-183/96/182 biogenesis by nuclear PP1. miR-183/96/182 are produced by the canonical pathway of miRNA biogenesis, involving transcription through RNA polymerase II into a single pri-mir-183-96-182 (1), which is further processed by Drosha/DGCR8 microprocessor complex into three different precursors, pre-mir-183, -96, -182 (2). These precursors are exported out into the cytoplasm through exportin 5 (3) and further processed by Dicer into mature miRNAs (4). Activated phospho-SMAD 1/5 localize to the nucleus to preferentially increase the micorprocessing of pri-mir-183-96-182 by binding to the SMAD-binding element present on its terminal loop, and microprocessor accessory component p68 (5). Increase in nuclear PP1 activity with aging and/or with TDP-43 loss of nuclear function, dephosphorylates SMAD 1/5, thus impairing their interaction with microprocessor accessory component p68 and pri-mir-183-96-182, resulting in decreased biogenesis of miR-183/96/182 (6).

3.7. Supplementary Figures

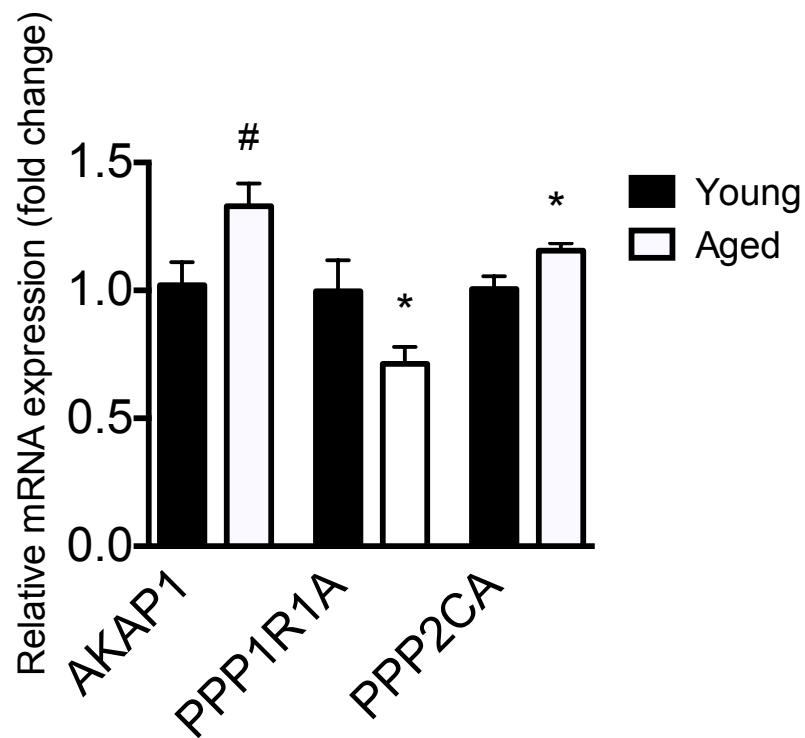
a. Weak novel object recognition (NOR) protocol and setup



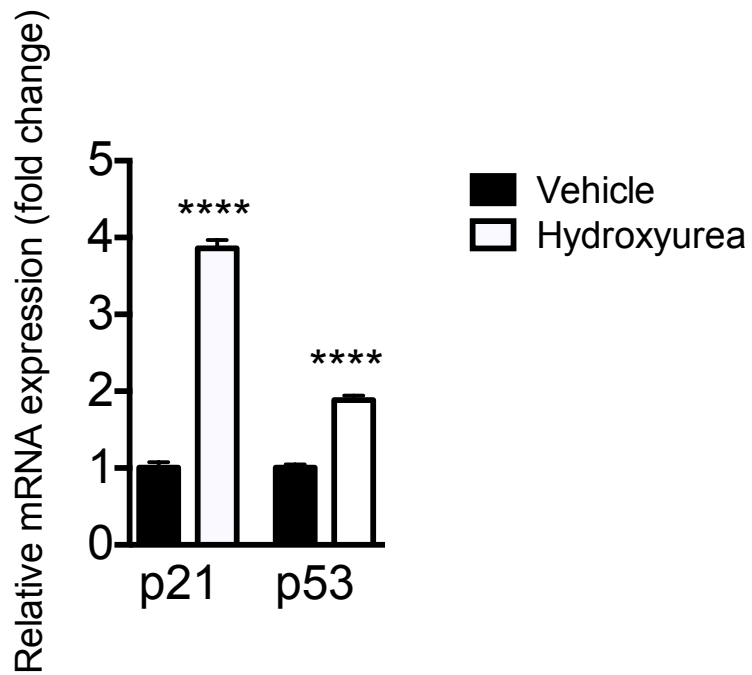
b. Object exploration followed by miRNA quantification



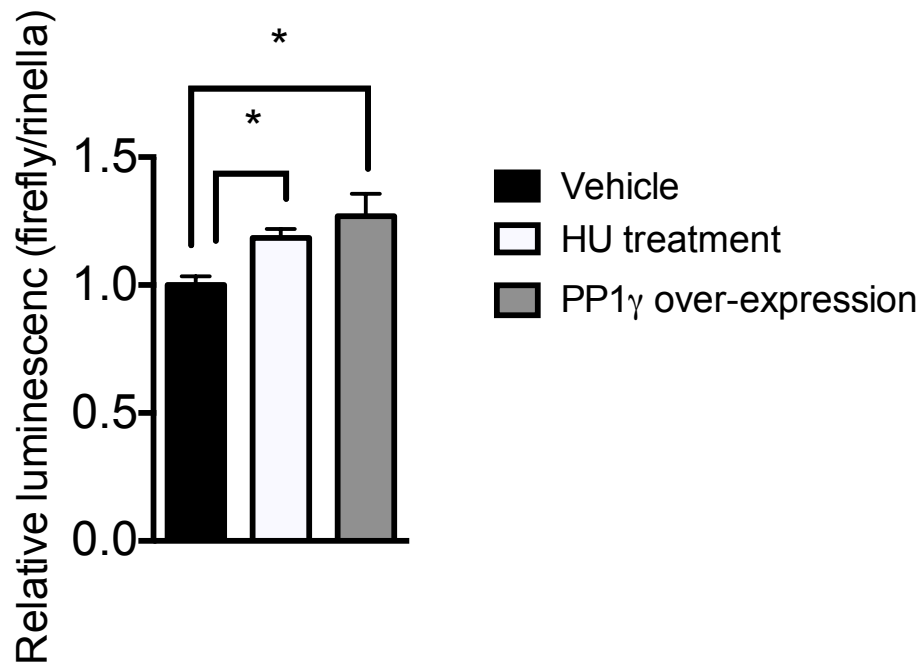
Supplementary Fig. 1: Novel object recognition (NOR) training of mice (a) Experimental set-up of NOR training, followed by memory testing defined by discrimination for the novel object, performed at 24 hours and 48 hours post-training. (b) Experimental set-up of object exploration conducted prior to tissue collection for analysis of miR-183/96/182 expression in the hippocampus.



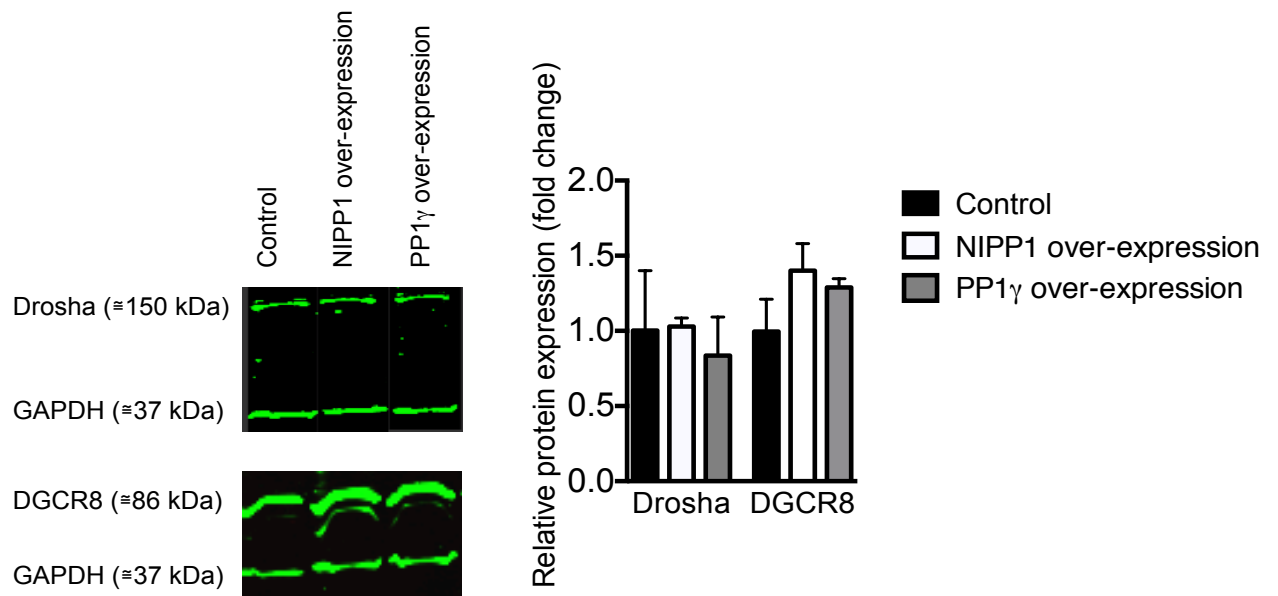
Supplementary Fig. 2: Altered expression of PP1 regulators with aging. Hippocampal tissue collected from aged (20-21 months) mice showed an increased expression of AKAP1 and PP2CA, and decreased expression of PPP1R1A compared to young (4-5 months old) mice (unpaired t test: AKAP1: unpaired t test, # $p < 0.1$; PPP1R1A: * $p < 0.05$; PP2CA: * $p < 0.05$). Young, $n=6$, aged, $n=6$.



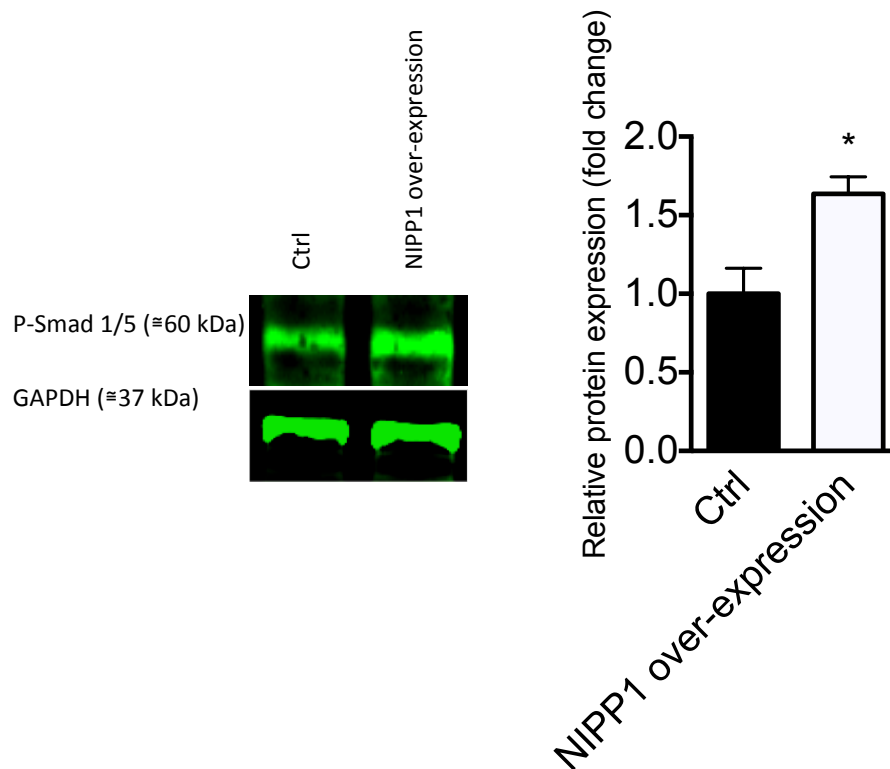
Supplementary Fig. 3: Hydroxyurea (HU) induces cellular senescence in mouse neuroblastoma (N2a) cells. Treating N2a cells with 8mM HU increased the mRNA expression of senescent markers p21 and p53 compared to vehicle treatment (unpaired t test: p21: **** $p < 0.0001$; p53: **** $p < 0.0001$). Vehicle, $n=4$, HU, $n=4$.



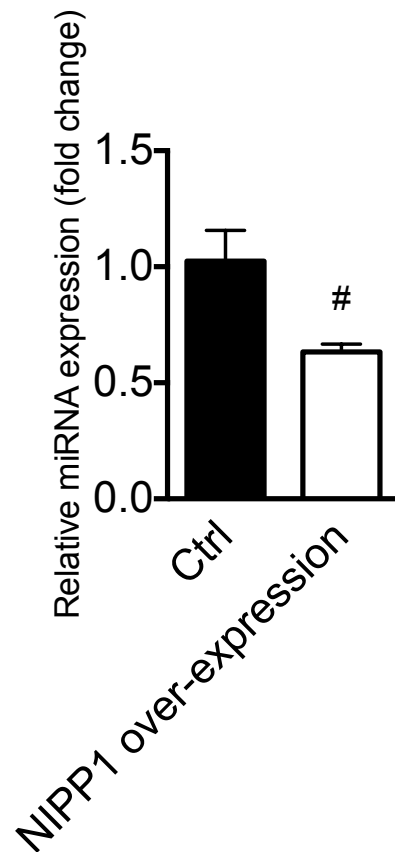
Supplementary Fig. 4: Decreased microprocessor processing of pri-mir-183, expressed as increased firefly/renilla luminescence, on HU-induced senescence, as well as, PP1 γ over-expression in N2a cells (one-way ANOVA, $F_{2,9}=5.772$, $p=0.02$, post-hoc: HU $*p<0.05$, PP1 γ $*p<0.05$). Vehicle, $n=4$, HU, $n=4$, PP1 γ over-expression, $n=4$.



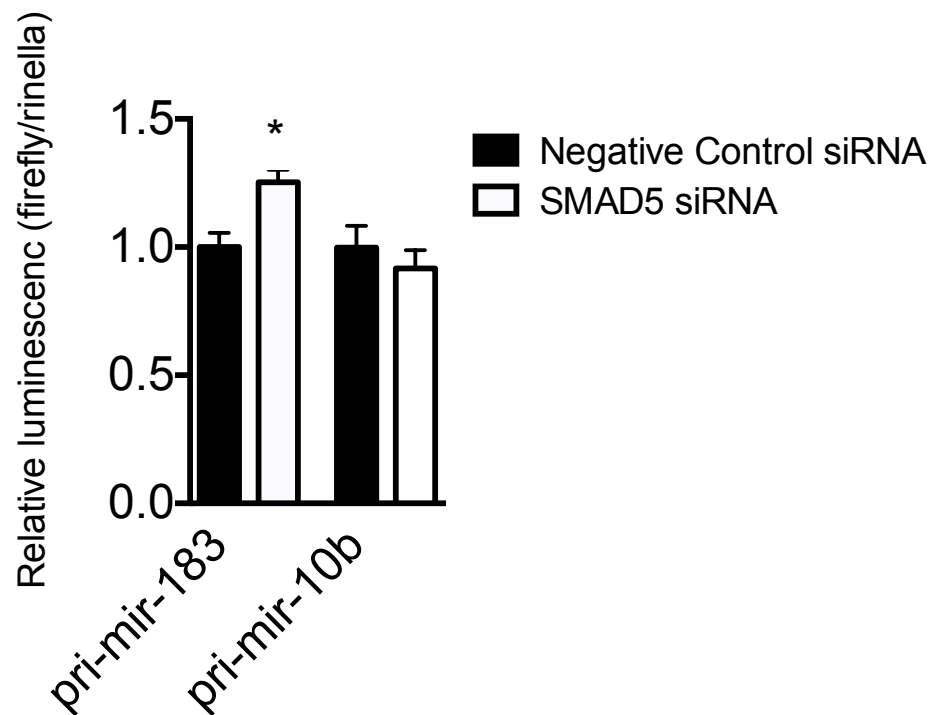
Supplementary Fig. 5: Nuclear PP1 does not regulate the expression of Drosha/DGCR8. Representative blots (left) and quantification (right) of Drosha and DGCR8 protein in the N2a cells after NIPP1 and PP1 γ over-expression. Drosha (one-way ANOVA, $F_{2,5}=0.215$, $p=0.814$), DGCR8 (one-way ANOVA, $F_{2,5}=2.016$, $p=0.248$). Control, $n=3$, NIPP1 over-expression, $n=3$, PP1 γ over-expression, $n=3$.



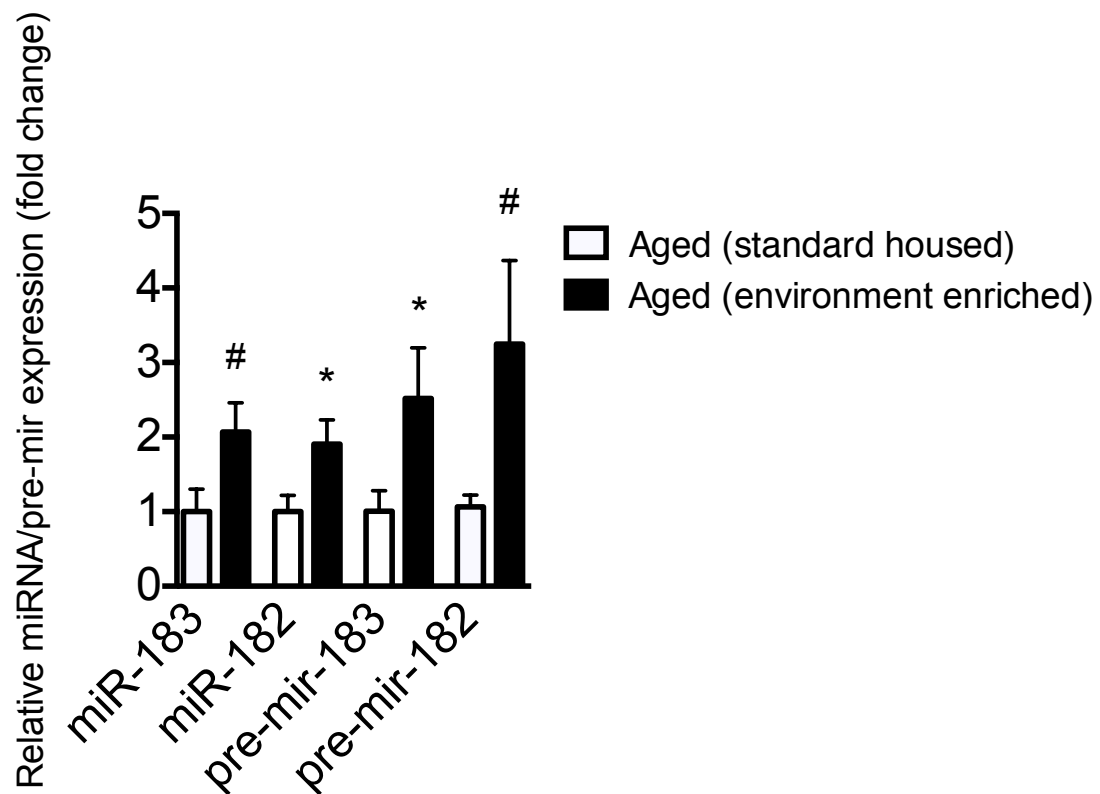
Supplementary Fig. 7: Nuclear PP1 regulates SMAD 1/5 phosphorylation. Representative blot (left) and quantification (right) of phospho-SMAD1/5 in N2a cells after NIPP1 over-expression showing an increase in their protein expression (unpaired t test: $*p < 0.05$). Control, $n=3$, NIPP1 over-expression. $n=3$.



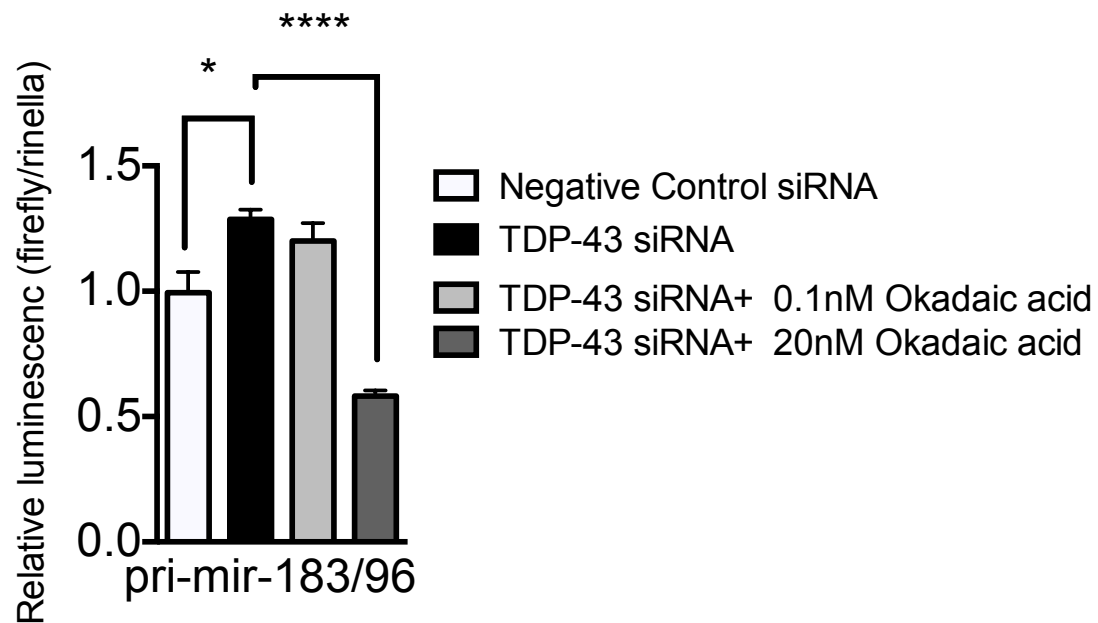
Supplementary Fig. 8: PP1 regulates SMAD-dependent miRNA. miR-21, a miRNA regulated at the microprocessor level by R-SMAD, is non-significantly decreased in N2a cells on NIPP1 over-expression (unpaired t test: # $p < 0.1$). Control, $n=4$, NIPP1 over-expression, $n=4$.



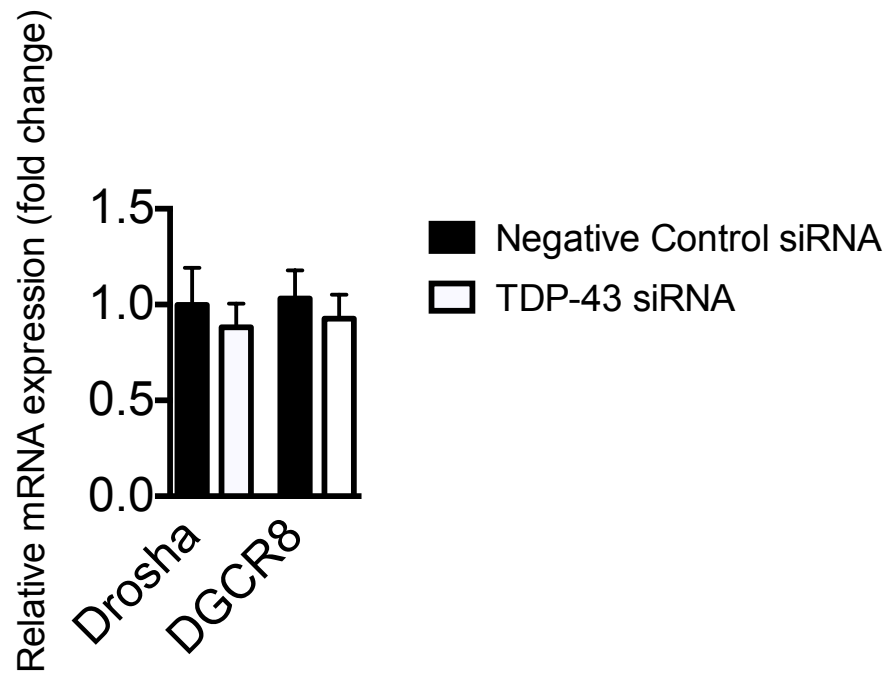
Supplementary Fig. 9: SMAD5 selectively regulates microprocessor processing of pri-mir-183/96. Decreased microprocessor processing of pri-mir-183, expressed as increased firefly/renilla luminescence ratio, but not of a control miRNA pri-mir-10b, on SMAD5 knock down in N2a cells (unpaired t test: pri-mir-183: * $p < 0.05$; pri-mir-10b: $p = 0.487$). Negative control siRNA, $n = 4$, SMAD5 siRNA, $n = 4$.



Supplementary Fig. 12: Environment enrichment (EE) in aged (19-21 months old) mice upregulates miR-183/182, as well, as their precursors. Increased expression of miR-183 and -182, and their precursors in hippocampal tissues collected from aged mice treated with four weeks of EE compared to aged mice with standard housing (unpaired t test: miR-183: # $p < 0.1$; miR-182: * $p < 0.05$; pre-mir-183: # $p < 0.1$; pre-mir-182: * $p < 0.05$). Aged (standard-housed), $n=6$, aged (environment enriched), $n=6$.



Supplementary Figure 14: Effect of TDP-43 on microprocessor processing of pri-mir-183/96 is PP1 dependent. Decreased microprocessor processing of pri-mir-183/96 on TDP-43 knock down is reversed in the presence of 20 nM (inhibits PP1/PP2A), but not with 0.1nM Okadaic acid (inhibits PP2A) (one-way ANOVA: $F_{3,10}=27.55$, $p<0.0001$, post-hoc: siRNA TDP-43 $*p<0.05$, siRNA TDP-43+ 20nM Okadaic acid $****p<0.0001$). Negative control siRNA, $n=4$, TDP-43 siRNA, $n=3$, TDP-43 siRNA+0.1 nM Okadaic acid, $n=3$, TDP-43 siRNA+20nM Okadaic acid, $n=4$)



Supplementary Figure 15: Unchanged mRNA expression of Drosha and DGCR8 on TDP-43 knock-down compared to a negative control (unpaired t test: Drosha: $p=0.631$; DGCR8: $p=0.605$). Negative control siRNA, $n=4$, TDP-43 siRNA, $n=4$.

3.8. References

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4. Discussion

Our findings newly reveal a role for miRNA cluster miR-183/96/182 in formation of long-term memory (LTM) in mammals. They further show that nuclear protein phosphatase 1 (PP1) regulates miR-183/96/182 biogenesis to fine-tune their expression upon learning. Importantly, age-related cognitive decline (ARCD) and cognitive dysfunction associated with TDP-43 proteinopathies (CD TDP) involve an increase in nuclear PP1 activity and consequential impairment in the biogenesis of miR-183/96/182.

4.1. Hippocampal miR-183/96/182 regulate memory

Our study is the first investigation showing a collective role for miRNA cluster 183/96/182 in formation of LTM. However, individually miR-182 has been previously implicated in amygdala-dependent fear memory (Griggs et al. 2013). Griggs et al. showed that miR-182 is down regulated in mouse lateral amygdala (LA) after auditory fear conditioning. Importantly, over-expression of miR-182 in mouse LA disrupted fear memory (Griggs et al. 2013). This is in contrast to our study, where we observed an increase in hippocampal miR-182, along with its partners miR-183 and miR-96 upon learning. There are several reasons, which could have lead to a disparity between these two studies. First, there are differences in the molecular/structural underpinnings of fear versus object recognition memory. For example, fear memory shows a greater dependency on the structural connectivity between different cortical hemispheres compared to object recognition memory according to studies on mice with agenesis of the corpus callosum (MacPherson et al. 2008). Second, it is possible that the functionality and regulation of miR-182 are different in different neuroanatomical regions. miRNA expression is known to complement the expression of their targets (Ha & Kim, 2014), and it is possible that the targets of miR-182 are differentially expressed in different brain regions, necessitating the need for comparative baseline expression of miR-182. For example, BDNF, a known target of miR-182 is decreased in area CA3 of hippocampus, but increased in basolateral amygdala in mice after chronic immobility stress (Lakshminarasimhan & Chatterji, 2012). It is possible that the stressful response elicited during auditory fear conditioning increases BDNF in LA of mice, necessitating a decrease in LA miR-182, whereas, the memory

component of auditory fear conditioning still increases miR-182 in the hippocampus. Similarly, Rac1, a target of miR-182, which is increased in mouse LA according to Griggs et al. is decreased in the hippocampus after spaced fear training in rats (Jiang et al. 2016). Finally, recently it has been pointed out that the last member of miRNA clusters can be subjected to different post-transcriptional regulation than the other members under specific conditions (Du et al. 2015). Hence, it is possible that the disparate expression of miR-182 in mouse LA and hippocampus in these two classical memory paradigms will not extend to other members of the cluster, i.e., miR-183 and miR-96.

MiR-183/96/182 were previously shown to exhibit an activity-dependent turnover in the mouse retinal cells (Krol et al. 2010). We show that this dynamic turnover characteristic of miR-183/96/182 also extends to neurons. The level of mature miR-183/96/182 immediately decreases with neuronal activity in N2a cells. However, this early decrease is followed by a subsequent increase on long-term stimulation. This subsequent increase in their expression is blocked when N2a cells are treated with transcriptional inhibitor Actinomycin D, suggesting that neuronal activity leads to a rapid usage of the pool of available miR-183/96/182, which are then replenished through transcriptional increase in their biogenesis. Importantly, inhibition of nuclear PP1 restocks the pool of mature miR-183/96/182 in the absence of transcription by increasing their post-transcriptional processing through the microprocessor complex. This may become vital in conditions, where the physiological transcriptional increase in miR-183/96/182 upon neuronal activity is sub-optimal. For instance, aging is known to decrease global transcription in the brain (Park et al. 2015), possibly by decreasing the activity of transcription factors, such as NF- κ B (Southwork et al. 2009). Similarly, high levels of oxidative stress inhibit gene transcription, partly through changes in histone acetylation (Berthiaume et al. 2006). Indeed, both these conditions are associated with impairment in multiple cognitive domains in mammals (Fukui et al. 2002). Artificially increasing hippocampal level of miR-183/96/182, therefore, becomes a potential therapeutic candidate to rescue cognitive deficits associated with such conditions. ARCD was indeed partially reversed by over-expression of miR-183/96/182 in this study. In future, it will be

interesting to investigate if miR-183/96/182 have the potential to rescue the cognitive deterioration associated with conditions of high oxidative stress in the brain, such as ischemic stroke (Nathaniel et al. 2015) and traumatic brain injury (Rodriquez-Rodriquez et al. 2014).

The bi-directional effect of miR-183/96/182 in this study strongly establishes their role in regulation of hippocampus-dependent memory. Artificial over-expression of miR-183/96/182 in mouse hippocampus enhanced, whereas, their hippocampal inhibition impaired, LTM after a weak protocol of novel object recognition (NOR) training. These effects are likely attributed to a combinatorial action of multiple targets of miR-183/96/182. Indeed, we found significant decrease in various targets of miR-183/96/182 in mice over-expressing the cluster. One such target Neuregeulin 1 (NRG1) regulates the intricate balance between excitatory and inhibitory connections within the brain, and its dysfunction is implicated in schizophrenia (Agarwal et al. 2014). Two other targets, GRM5 and PP2CA are associated with ARCD (Ménard & Quirion, 2012) and Alzheimer disease (Sontag & Sontag, 2014) respectively. Similarly, NUFIP2 is linked to Fragile X intellectual disability (Bardoni et al. 1999). Finally, another target is HDAC9, a histone deacetylase, whose inhibition enhances cognition in mice (Gräff et al. 2013).

To show that manipulation of miR-183/96/182 targets can recapitulate the phenotypic effects of miR-183/96/182 over-expression/inhibition, we chose HDAC9 for further analysis. After validating a direct interaction between miR-182 and HDAC9 through luciferase assays, we interfered with miR-182 binding to HDAC9 *in vivo* through locked nucleic acid (LNA) technology based target-site blockers. This interference impaired object exploration in mice, without an appreciable effect in LTM. This result is not unexpected, considering only one target of miR-183/96/182 was blocked. Multiple targets of a miRNA are sometimes functionally redundant (Allen et al. 2007), requiring their combinatorial suppression by the miRNA to produce a detectable phenotype. HDAC9 is even more unique in this case as it belongs to a family of enzymes known to be functionally identical (Haberland et al. 2009). Hence, it is likely that HDAC9 manipulation did not produce a cognitive phenocopy of miR-183/96/182 inhibition due to a compensatory contribution from other miR-

183/96/182 targets and/or HDACs. Regardless, interfering with HDAC9 impaired exploratory behavior in mice, which is a crucial pre-requisite of LTM formation. This suggests that the cognitive phenotype of hippocampal miR-183/96/182 is mediated, in part, through HDAC9.

Bioinformatics analysis using DIANA tools miR-path software on miR-183/96/182 targets reveals their involvement in multiple molecular pathways associated with cognitive functioning (Table 1).

Table 4.1: Biological pathways potentially regulated by miR-183/96/182

miRNA	Five most affected pathways
miR-183	Transcriptional regulation , Wnt signaling, dopaminergic neurotransmission, glycan degradation, tight junctions
miR-96	Regulation of actin cytoskeleton , MAPK signaling , prostate cancer, axon transport , long-term potentiation
miR-182	Long-term depression , neurotrophin signaling , bacterial invasion of epithelial cells, oocyte meiosis, long-term potentiation

Our investigation provides experimental evidence in support of the bioinformatics analysis by revealing transcriptional regulator HDAC9 as a validated target of miR-183/182. Similarly, two other targets found decreased in mouse hippocampus after miR-183/96/182 over-expression, NGR1 and Protein kinase C zeta (PRKCZ) (Kwon et al. 2008, Sackter, 2008), have been invariably reported to regulate LTP and LTD. In future investigations, it will be crucial to study LTP and LTP in mice with hippocampal over-expression and inhibition of miR-183/96/182.

Further to LTP/LTD, another molecular pathway likely implicated in mediating the cognitive effects of miR-183/96/182 is adult neurogenesis, which is increasingly associated with memory formation and re-consolidation in mammals (Suarez-Pereira & Carrion, 2015). Importantly, the expression and activity of HDAC9 is uniquely suppressed in adult neural stem cells through all stages of neural differentiation, allowing an expression exclusively in post-mitotic neurons (Lang et al. 2012). Expression of miR-183/96/182 and their targets in neural stem cells, as well as, their contribution to adult hippocampal

neurogenesis for memory formation and re-consolidation may make an important subject for future investigations.

Finally, miR-183/96/182 may regulate memory through their control of DNA repair mechanisms (Krishnan et al. 2013). Activity-induced DNA breaks have been identified as a key mechanism governing expression of immediate early genes (IEGs) in the mouse brain (Madabhushi et al. 2015). Considering the critical role of IEGs in memory formation, it is possible that miR-183/96/182 may make an important contribution to this pathway by suppressing DNA repair mechanisms, thus allowing persistent activity-induced DNA breaks.

These predicted and validated roles of miR-183/96/182 in key regulatory hubs controlling cognitive functions in mammals make them attractive therapeutic candidates for treatment of cognitive disorders. MiRNA-based therapeutics, involving both gain- and loss-of-miRNA-function are being increasingly attempted in the cancer field (Woldemichael & Mansuy, 2016). However, a widespread and effective use of miRNA therapeutics has been hampered due to the limited cellular permeability of miRNA modulating oligonucleotides owing to their large size and negatively charged backbone (Schmidt, 2014). This problem could be even more pertinent for miR-183/96/182 delivery in the brain due to the impeding blood-brain barrier. In recent years, drug formulations that overcome these challenges by packaging the oligonucleotide inside ionizable liposomal nanoparticles or through LNA-modification of negative charged backbones have been developed for some miRNAs (Bader et al., 2011). In future, it will be interesting to see if these innovative strategies can increase the brain uptake and penetration of miR-183/96/182 allowing their targeted delivery to specific brain regions of interest.

Another challenge related to the therapeutic potential of miR-183/96/182 is their widespread influence on gene regulation, which could lead to undesirable effects. For instance, a recent study has revealed that miR-182 overexpression in mouse hippocampus may lead to pro-depressive behaviors (Li et al. 2015). This could be a possible off-target effect as artificial miRNA overexpression can perturb several target genes, and may also trigger multiple compensatory pathways. One means of tackling this challenge could be

meticulous analysis of miRNA targets implicated in cognition versus depression, followed by selective disruption or enhancement of interaction between miR-183/96/182 and their specific memory-relevant targets through LNA target site blockers.

4.2. PP1 regulates the biogenesis of miR-183/96/182

MiR-183/96/182 are produced through the canonical pathway of miRNA biogenesis involving transcription of pri-mirs, their processing into pre-mirs through the Drosha-DGCR8 microprocessor in the nucleus, export into the cytoplasm, and conversion into mature miRs by Dicer (Ha & Kim, 2014). Based on the observation that miR-183/96/182 are up regulated in the hippocampus of NIPPI* mice, we questioned how nuclear PP1 regulates the nuclear biogenesis of these miRNAs. Early biogenesis of miR-183/96/182 could be regulated by PP1 for a number of reasons. PP1 can regulate the occupancy of RNA pol II on the promoter region of a number of genes, for example, CREB and NF- κ B, thereby regulating their transcription (Koshibu et al. 2009). CREB and NF- κ B, themselves, regulate the transcription of many miRNAs (Taganov et al. 2006, Nudelman et al. 2010). Further, activity of RNA pol II can be altered by PP1 through dephosphorylation of its C-terminal domain (Hirose & Ohkuma, 2007). Beyond this transcriptional control, PP1 could also regulate the biogenesis of miR-183/96/182 post-transcriptionally through regulating the expression, sub-cellular localization, and activity of major components of the nuclear microprocessor (Tang et al. 2010, Tang et al. 2011, Herbert et al. 2013).

By using transcriptional silencing assays, we very judiciously demonstrated that regulation of miR-183/96/182 is transcription-independent. A transcriptional-dependence would have lead to a reversal of pre-mir-183/96/182 up regulation on inhibition of nuclear PP1 in the absence of transcription. Contrary to this, we observed a decrease in the pri-mir-183-96-182 and a persistent up regulation of pre-183/96/182 when nuclear isoform of PP1, PP1 γ was knocked down even with transcriptional silencing. This was complemented by the results of neuronal activity assays, which revealed that PP1 γ knock down replenished the mature miR-183/96/182 in the absence of

transcription. Together, these results established that PP1 regulates miR-183/96/182 biogenesis post-transcriptionally.

We next investigated if nuclear PP1 regulates Drosha-DGCR8 microprocessor complex. Neither the expression nor the sub-cellular localization of Drosha/DGCR8 was altered on manipulating nuclear PP1 in our study. Importantly, while microprocessor processing of artificially introduced pri-mir-183/96 and pri-mir-182 was enhanced on nuclear inhibition of PP1 in N2a cells, microprocessor-mediated biogenesis of a control miRNA miR-10b was unperturbed. These mechanistic studies indicate that regulation of the microprocessor-mediated biogenesis of miRNAs by nuclear PP1 is selective for miR-183/96/182. Indeed, only a hand full of miRNAs were differentially regulated in the hippocampus upon NIPP1* induction in mice, which is in agreement with the *in vitro* microprocessor activity assays.

The intriguing finding that nuclear PP1 can selectively regulate the microprocessor-mediated biogenesis of miR-183/96/182 is an important addition to the repertoire of evidence supporting the notion that activity of microprocessor can be selectively regulated by certain factors and/or under certain conditions (reviewed in Ha & Kim, 2014). As mentioned previously, a majority of these 'microprocessor regulators' include RNA binding proteins, which either bind to certain pri-mirs and preferentially increase or decrease their cleavage by the microprocessor, or interfere with the binding of Drosha and DGCR8 (Ha & Kim, 2014). Of particular interest to us among these 'microprocessor' regulators were MeCP2 and R-SMADs, as their nuclear expression and activity depend on phosphorylation, making them vulnerable to changes in nuclear PP1 (Li et al. 2014, Wrighton et al. 2009). Using Western-blot based phosphorylation assays, we identified that phosphorylation of SMAD 1/5 can be regulated by nuclear PP1 (Chapter 3), whereas, MeCP2 phosphorylation did not vary with increase in nuclear PP1 (Fig. 4.1 left). Interestingly, SMAD 5 mRNA expression, along with that of KSRP, was regulated by nuclear PP1 as well (Fig. 4.1 right).

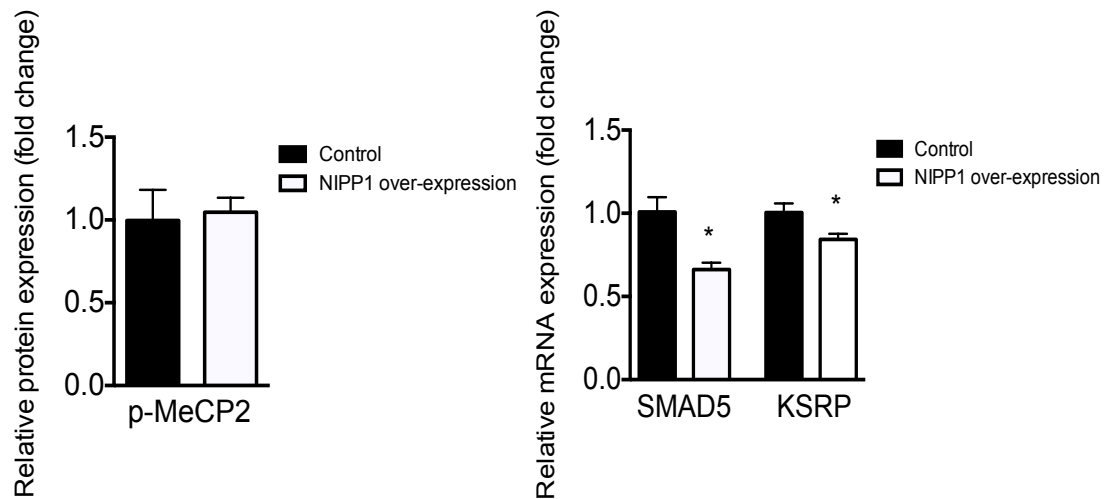


Fig. 4.1. Nuclear PP1 controls the expression of known microprocessor regulators. (left) NIPP1 over-expression did not alter the protein expression of phospho-MeCP2 in the nuclear fraction of mouse N2a cells (unpaired t test: $p=0.819$). (right) NIPP1 over-expression decreased the mRNA expression of SMAD-5 and KSRP in mouse N2a cells (unpaired t test: SMAD-5: $p<0.05$; KSRP: $*p<0.05$). Control, $n=4$, NIPP1 over-expression, $n=4$.

SMAD proteins are down-stream signaling molecules of transforming growth factor- beta (TGF- β) and bone morphogenic protein (BMP), which translocate to the nucleus on activity-induced phosphorylation. Together, with SMAD-4 (also known as co-SMAD), R-SMADs control the expression of multiple genes transcriptionally (reviewed in Massgu , 2012). However, the regulation of microprocessor by R-SMADs is independent of co-SMAD, and involves their interaction with microprocessor accessory protein p68 (Davis et al. 2008, Davis et al. 2010, Blahna & Hata, 2012). Control of nuclear SMAD 1/5 phosphorylation by PP1, combined with their known role in selectively regulating the microprocessor processing, makes them a likely mediator for the selective regulation of miR-183/96/182 by nuclear PP1. This possibility was verified through multiple modalities: 1) a confirmation of the selective effect of SMAD 5 on microprocessor processing of pri-mir-183 and pri-mir-182, similar to their regulation by nuclear PP1, 2) altered expression of miR-21, a SMAD-dependent miRNA in N2a cells after PP1 manipulation, and 3) bio-informatics validation of presence of SMAD binding element on pri-mir-183-96-182. Taken together, these results strongly suggest R-SMADs as intermediates between nuclear PP1 and microprocessor processing of pri-mir-183-96-182 (Fig. 4.2).

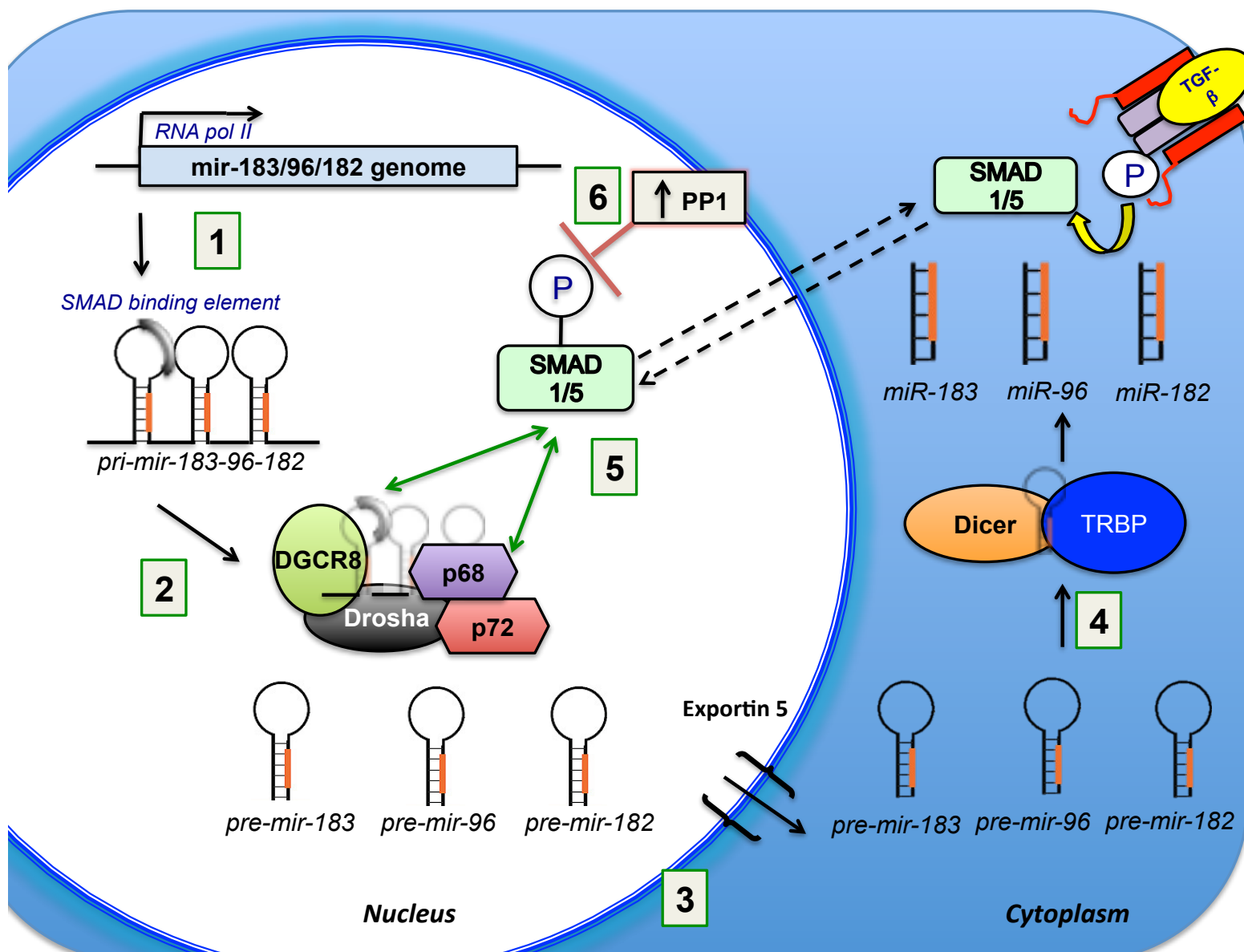


Fig.4.2. Model representing regulation of miR-183/96/182 biogenesis by nuclear PP1. miR-183/96/182 are produced by the canonical pathway of miRNA biogenesis, involving transcription through RNA polymerase II into a single *pri-mir-183-96-182* (1), which is further processed by *Drosha/DGCR8* microprocessor complex into three different precursors, *pre-mir-183*, *-96*, *-182* (2). These precursors are exported out into the cytoplasm through *exportin 5* (3) and further processed by *Dicer* into mature miRNAs (4). Activated phospho-*SMAD 1/5* localize to the nucleus to preferentially increase the microprocessing of *pri-mir-183-96-182* by binding to the *SMAD-binding element* present on its terminal loop, and the microprocessor accessory component *p68* (5). Increase in nuclear *PP1* activity dephosphorylates *SMAD 1/5*, thus impairing their interaction with microprocessor accessory component *p68* and *pri-mir-183-96-182*, resulting in decreased biogenesis of miR-183/96/182 (6).

Importantly, all the different components of this regulatory pathway depicted above have been linked with cognitive functions in mammals. While the role of PP1 as a negative regulator of memory is well established (Genoux et al. 2002), deletion of DGCR8 was shown to impair neurogenesis and working memory in mice (Ouchi et al. 2013). Similarly, TGF- β and down-stream SMAD signaling are essential for maintenance of LTP and hippocampal-dependent memory in mice (Caraci et al. 2015). Finally, our study has established a crucial down-stream role for miR-183/96/182 in formation of long-term memory in mice.

Experimental evidence supporting this 'nuclear PP1 \rightarrow R-SMAD \rightarrow Drosha/DGCR8 microprocessor \rightarrow miR/183-/96-/182' pathway could benefit from investigations confirming a direct interaction of pri-mir-183-96-182 with SMAD-5 through cross-linking immunoprecipitation (CLIP) analysis. Such analysis is expected to reveal an increased interaction of pri-mir-183-96-182 with SMAD-5 and p68 complex on inhibition of nuclear PP1.

From a therapeutic point of view, each component of this pathway has considerable potential to be employed for cognitive enhancement. Spaced learning; a known memory-enhancing paradigm, mediates its effects through inhibition of PP1 (Genoux et al. 2002, Waddell, 2003). In our study, spaced learning employed during strong NOR training protocol paralleled the effect of NIPP1* over-expression on memory formation, as well as, miR-183/96/182 expression in mice. Importantly, spaced learning in NIPP1* mice did not have any additional effect on either their memory performance or their hippocampal miR-183/96/182 expression (unpublished data). Further, artificial over-expression of miR-183/96/182 enhanced memory only in the weak NOR protocol, which does not employ spaced learning. These results reiterate that PP1 has a constraining role on memory formation, which can be physiologically removed through spaced learning paradigms and/or artificially over-expressing its down-stream effectors, miR-183/96/182. Interestingly, *bacopa monneira*, a traditional cognitive enhancer was recently shown to enhance long-term memory in rats through decrease in hippocampal PP1 and HDAC9, a target of miR-183/96/182 (Preethi et al. 2014). Similarly, mild

increase in ALK-5 dependent TGF- β signaling improves hippocampal-dependent spatial memory through promoting late-adult neurogenesis (He et al. 2014)

From a clinical perspective, further dissecting the regulation of miR-183/96/182 by nuclear PP1, also in other learning paradigms, will not only reveal promising targets for memory disorders, but may also provide valuable clues in understanding the unique memory phenotype of post-traumatic stress disorder (PTSD). PTSD patients experience an exaggerated recall for traumatic memories accompanied by amnesia for the recognizable facts related to the traumatic event (Brewin & Holmes, 2003). Fear conditioning, a behavioral model for PTSD, decreases hippocampal miR-183/96/182 (unpublished data), which is contrary to their up regulation by NOR training. Similarly, Griggs et al. observed a decrease in amygdala miR-182 after auditory fear conditioning. It is possible that PP1 and miR-183/96/182 are differentially regulated in hippocampus versus amygdala during a traumatic event. Amygdala changes in PP1 and miR-183/96/182 during the traumatic event may underlie the exaggerated recall of traumatic memories, whereas the recognition memory could be impaired due to PP1 and miR-183/96/182 changes in the hippocampus. Dissecting these mechanisms further may aid in developing therapeutic strategies for PTSD.

4.3. Impaired PP1-dependent biogenesis of miRNA-183/96/182 underlies cognitive dysfunction associated with aging

The molecular mechanisms underlying ARCD have been proposed to involve protein phosphatases, in particular PP1 and CaN (Park et al. 2015). The results of our study confirm that increase in nuclear PP1 activity with aging underlies ARCD, and this effect is mediated through impaired nuclear biogenesis of miR-183/96/182.

There are multiple plausible reasons for the increase in nuclear PP1 activity with aging. The first possibility could be an increased expression of nuclear isoform of PP1, PP1 γ with aging. However, we found a comparable hippocampal expression of PP1 γ in aged versus young mice in our study

(unpublished data). Further to its expression, activity of PP1 can be regulated by >200 different proteins. These regulatory proteins target PP1 to different sub-cellular compartments, control its substrate specificity, as well as, bind to and block its phosphatase domains (reviewed in Munton et al. 2004). PP1 inhibitor PP1R1A was decreased, whereas, PP1-nuclear scaffolding partner AKAP1 was increased with aging in the mouse hippocampus in our study. Altered expression of these PP1 regulators with aging explain, in part, increased nuclear PP1 activity with aging. However, increased nuclear PP1 activity with aging likely implicates additional pathways. Off note, here is the evidence suggesting an altered balance between brain kinases and phosphatases with aging, leading to a dramatic increase in phosphatases (Norris et al. 1998). The molecular underpinnings of this aging-induced switch are not entirely clear, but may involve altered neuronal Calcium homeostasis (Toescu & Verkhatsky, 2000, Oh et al. 2013). Aging increases Calcium in neurons dually, through an increased Calcium influx across the plasma membrane, as well as, a reduced intracellular Calcium buffering (Oh et al. 2013). Intracellular influx of Calcium may then activate PP1 through dephosphorylating its C-terminus (Hou et al. 2013). However, this hypothesis is purely speculative at this time and could benefit from more direct experimental evidence.

Importantly, our *in vivo* data revealed a post-transcriptional blockade in the up regulation of miR-183/96/182 upon learning in aged mice, combined with an increase in nuclear PP1 activity in the mouse hippocampus with aging. To validate the hypothesis that aging-induced increase in nuclear PP1 activity impairs the microprocessor processing of miR-183/96/182, we took advantage of an *in vitro* model of neuronal aging. This model is based on the use of chemotherapeutic agent hydroxyurea (HU) to induce cellular senescence in neural stem cells (Dong et al. 2014). We adapted HU to our *in vitro* model, and successfully induced cellular senescence in N2a cells, which was verified through an increased expression of senescent markers p21 and p53. Intriguingly, HU treatment impaired the microprocessor processing of pri-mir-183/96, but not of pri-mir-182. This finding is in slight contrast to an equivocal effect of PP1 on both pri-mir-183/96 and pri-mir-182 processing. This peculiar regulation of pri-mir-182 processing on induction of cellular senescence could

be attributable to the recently revealed anomalous processing dynamics of the last member of miRNA clusters. The biogenesis of last member of miRNA clusters may involve an additional precursor form, 'pro-miRNA', under certain conditions (Du et al. 2015). Whether miR-182 biogenesis involves this additional precursor form, which may be regulated differently from the other members of the cluster, is an important question warranting further investigations.

Virus-mediated hippocampal over-expression of miR-183/96/182 reversed ARCD in this study. Aged mice were stereotaxically injected in their hippocampus with a self-complementary adeno-associated viral vector expressing pre-mir-183/96/182. Over-expressing pre-mir-183/96/182 ensured that the impaired microprocessor-mediated biogenesis of pre-mir-183,-96, and -182 was countered, but a normally functioning Dicer was still required for the eventual effect. This strategy conclusively established that the association between miR-183/96/182 and ARCD *specifically* involves regulation of their microprocessor processing by nuclear PP1, and ARCD can be corrected if this step is bisected even in the presence of increased nuclear PP1.

Up-stream inhibition of PP1 is another strategy to reverse ARCD, which is strongly supported by experimental evidence (Park et al. 2015). Environmental enrichment, an established memory-enhancing paradigm in aged rodents, decreased nuclear PP1 activity, and increased post-transcriptional biogenesis of miR-183/96/182 in our study. Besides this slow and persistent inhibition, rapid inhibition in the activity of nuclear PP1 with spaced learning also seems to transiently reverse ARCD. This is supported by the fact that aged rodents rarely show memory deficits when trained on the strong NOR protocol, which involves spaced learning, and is associated with inhibition of nuclear PP1 (Pavloupolos et al. 2013).

Taken together, these results highlight the potential of PP1- and miR-183/96/182-based modalities to reverse ARCD and/or to delay dementia in the elderly. Indeed, learning and efficient LTM formation was enhanced in a cohort of elderly humans with early AD using a strategy of spaced retrieval learning, an extrapolated human equivalent of spaced learning in mice (Jang et al.

2015). Similarly, multi-modal stimulation, an extrapolated equivalent of environment enrichment, has shown promising results in reducing dementia symptoms in elderly humans with AD and minimal cognitive impairment (Luttenberger et al. 2012, Dannhauser et al. 2014). Finally, while miR-183/96/182 based therapeutics seem infeasible for application in humans at this point, drugs selectively modulating their targets are available, and may make attractive candidates for treatment of ARCD. HDAC inhibitors have shown considerable promise in this regard (Gräff et al. 2013).

4.4. Impaired PP1-dependent biogenesis of microRNA-183/96/182 underlies cognitive dysfunction associated with TDP-43 proteinopathies

Cognitive impairment is a hallmark feature of multiple neurodegenerative conditions, believed to be the result of neuronal death in the brain regions governing cognitive functions (Weintraub & Mesulam, 2009). While neuronal death provides a coherent mechanism for late deterioration of cognitive functions in neurodegenerative disorders, it does not explain the early progressive impairment of memory observed in these conditions. Such impairments often precede detectable neuronal death, and are rarely amenable to pharmacological interventions (Rapp et al. 2002, Ford et al. 2015). Emerging evidence suggests that besides neuronal death, epigenetic mechanisms are responsible for early and persistent synaptic dysfunction and cognitive decline in the neurodegenerating brain (Gräff et al. 2012). These epigenetic changes include, notably, increased expression and activity of HDACs (Gräff et al. 2012, Stilling & Fischer, 2011). Our results suggest that impaired biogenesis of memory-promoting miRNAs could be an additional non-genomic blockade of the cognitive functioning in neurodegenerating disorders. Notably, loss of TDP-43, a postulated etiology underlying neurodegenerative ALS/FTLD, impaired the microprocessor-mediated biogenesis miR-183/96/182.

TDP-43 knockdown impaired the microprocessor processing of artificially introduced pri-mir-183 and pri-mir-182 in our *in vitro* microprocessor activity assays. Microprocessor activity can be controlled by TDP-43 through an increase in the stability of Drosha proteins *in vitro* (Di Carlo et al. 2013). Drosha/DGCR8 complex also interacts with TDP-43 in neuronal cell lines, where TDP-43 further binds to certain pri-mirs and differentially regulates their

processing by the microprocessor. These pri-mirs did not include pri-mir-183-96-182 (Kawahara et al. 2012). Our study newly adds miR-183/96/182 to the list of miRNAs regulated by TDP-43, in a PP1-dependent manner. Inhibition of PP1/PP2A by high-dose Okadaic treatment reversed the impairment in microprocessor processing of pri-mir-183/96 by PP1 knock down in our study. However, this impairment was not reversed when the cells were treated with low dose Okadaic acid, which only blocks PP2A (Ishihara et al. 1989). Taken together, these findings establish that TDP-43 regulates miR-183/96/182 biogenesis at the level of the microprocessor complex, through a PP1-mediated mechanism.

Importantly, TDP-43 knockdown increased nuclear PP1 activity in N2a cells. This effect is not surprising, as TDP-43 also regulates the activity of PP2A, a phosphatase closely related to PP1 (Perera et al. 2014). Increases in PP1 and PP2A with TDP-43 knockdown could also reflect a homeostatic response to an increase in TDP-dependent cyclin-dependent kinases (Ayala et al. 2008). While the exact molecular underpinnings of nuclear PP1 increase on TDP-43 knockdown remain elusive, they seem to involve mechanisms different from those observed in aging. Contrary to the observation in aged mice, analysis of human frontal cortex samples of ALS patients with TDP-43 pathology did not reveal alterations in the expression of PP1 regulatory sub-units. Instead, PP1 γ was increased at the mRNA level. The increase in this predominantly nuclear PP1 isoform could reflect changes in the nuclear splicosomal machinery, of which TDP-43 is an integral component, in ALS/FTLD brains (Conti et al. 2015).

Mounting evidence suggests that miRNA regulation by TDP-43 is multi-factorial, potentially affecting both the nuclear microprocessor, as well as, the cytoplasmic Dicer processing (Kawahara et al. 2012). While the regulation of the microprocessor by TDP-43 is specific to a sub-set of miRNAs, its control of Dicer seems more global. Disease-causing mutations in TDP-43 induce formation of stress granules, which interact with Dicer to inhibit its pre-miRNA processing activity (Emde et al. 2015). Thus, an effect of TDP-43 on Dicer activity could potentially confound our hypothesis about impaired microprocessor mediated biogenesis of miR-183/96/182 in TDP-43

proteinopathies. To exclude the contribution of Dicer, we quantified pre-mir-183/96/182, as well as, a control precursor pre-mir-10b in both nuclear and cytoplasmic RNA fractions extracted from the brain samples of ALS/FTLD patients. ALS/FTLD patients with TDP-43 pathology had decreased pre-mir-183/96/182, but an unperturbed pre-mir-10b in the nuclear RNA fraction. This result is in agreement with the hypothesis that TDP-43 loss of function in ALS/FTLD impairs the nuclear biogenesis of miR-183/96/182. On the other hand, both pre-mir-183/96/182 and pre-mir-10b were increased in the cytoplasmic fraction. Combined with a significant decrease of miR-183/96/182, and a non-significant decrease of miR-10b in ALS/FTLD brains, these results indicate a global impairment of Dicer-mediated miRNA biogenesis in TDP-43 proteinopathies.

Taken together, our results newly provide a novel mechanism for regulation of miRNA biogenesis by TDP-43 involving PP1, in addition to their previously known effects on Drosha and Dicer (Fig. 4.3). This effect is selective for miR-183/96/182 and could be a potential mediator of cognitive dysfunction observed in TDP-43 proteinopathies. Whether or not, decreased miR-183/96/182 contribute to the motoric phenotype of ALS is a question warranting further investigation. Although, there is evidence to suggest that miR-183 and miR-182 may have important roles in regulating muscular gene expression in animal models of pathological muscle atrophy (Kye et al. 2014, Hudson et al. 2014)

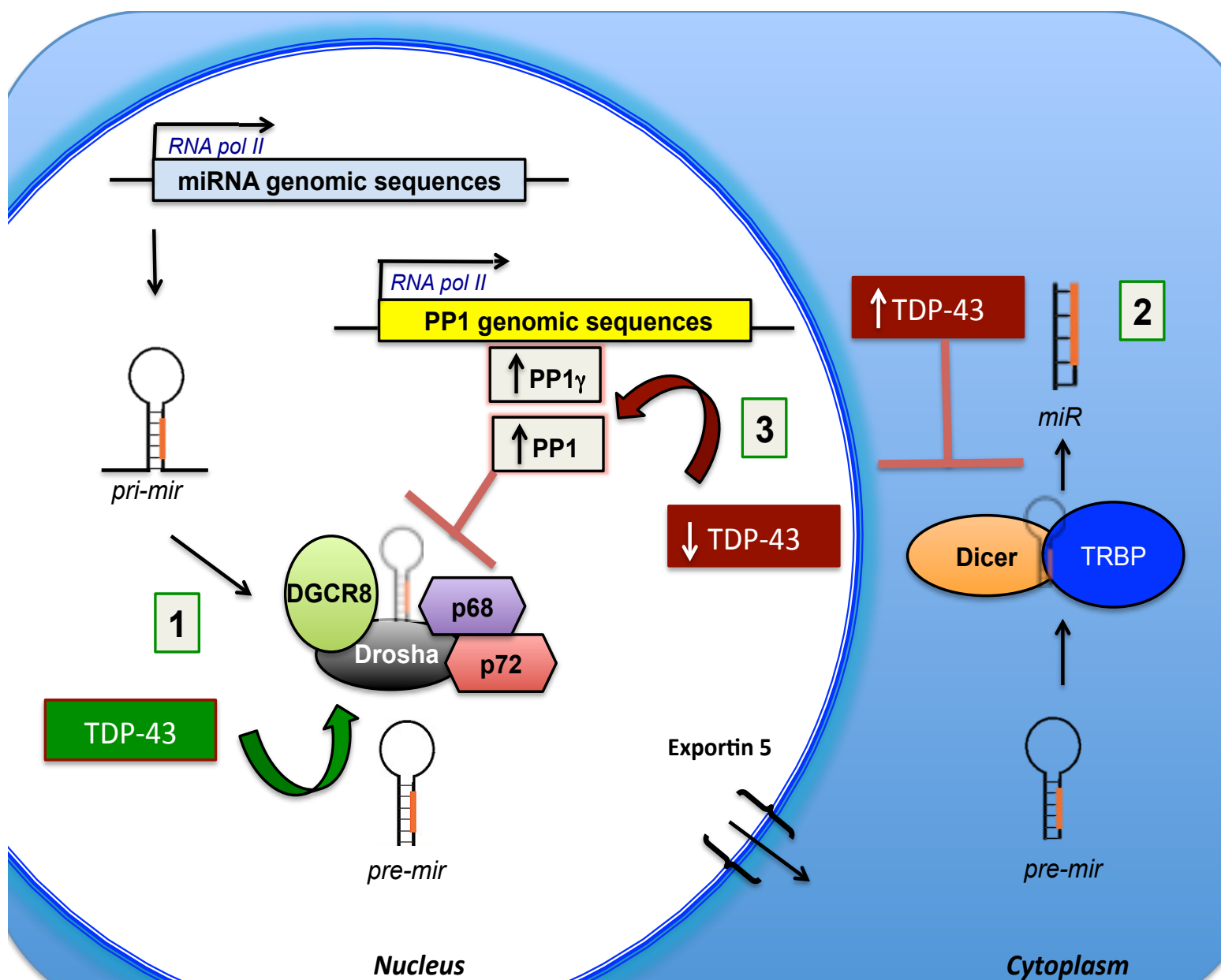


Fig. 4.3. Regulation of the microprocessor mediated miRNA biogenesis by TDP-43. Nuclear TDP-43 is known to increase the microprocessor processing during neuronal differentiation by stabilizing Drosha (1). Further, pathological cytoplasmic accumulation of TDP-43 impairs Dicer processing through unclear mechanisms (2). Our data shows that TDP-43 can additionally regulate biogenesis of certain miRNAs by regulation the expression of PP1 γ , predominantly nuclear isoform of PP1. TDP-43 loss of nuclear function increases nuclear PP1 activity through increased mRNA expression of PP1 γ , which impairs the microprocessor processing of miR-183/96/182 (3).

A decrease in miR-183/96/182 in ALS may also explain the unique neuropsychiatric phenotype associated with the disorder. ALS is associated with detectable cognitive impairment in around 50% of sporadic cases, which is

severe enough to be classified as dementia in roughly 15-20% cases (Ringholz et al. 2005). However, the prevalence of depressive symptoms in ALS patients is surprisingly low (<20%) in comparison to other neurodegenerative conditions (40-50%) (Rabkin et al. 2005). A report from another research groups suggested that miR-183/96/182 over-expression in mouse hippocampus promotes pro-depressive behavior in mice (Li et al. 2015). This contrasting effect of miR-183/96/183 on LTM formation versus behavior could provide a plausible explanation for decreased rate of depression in ALS patients, despite an increase in cognitive deficits and considerable physical disability.

Further, decreased miR-183/96/182 and their possible regulation by PP1 could explain the distinct disease-modifying effect of diabetes mellitus type 2 (DM2) on motor versus cognitive symptoms of ALS. While pre-morbid DM2 delays the onset of motor symptoms in ALS patients, it worsens their cognitive functioning (Jawaid et al. 2010). Notably, DM2 is associated with an increased PP1 activity in the skeletal muscles (Rastogi et al. 2003). A similar increase in PP1 activity in the brain with DM2 could lead to further suppression of miR-183/96/182 in ALS brains leading to worse cognitive functions, despite the beneficial effects of DM2 on the motor functions (Jawaid et al. 2010, Jawaid et al. 2013, Jawaid et al. 2015).

The results of this study highlight the potential of miR-183/96/182 and PP1-based therapeutics in treating CD TDP, similar to their effect on ARCD. However, the dual effect of TDP-43 on microprocessor, as well as, Dicer processing might require slight modifications to the miR-183/96/182 over-expression strategy we used for reversing ARCD. A viral vector expressing mature miR-183/96/182 or their mimics, thus bisecting the requirement of a functional Dicer, is more likely to have a cognitive enhancing effect for CD TDP.

4.5. General relevance of the results

The findings of this thesis are highly momentous broadly to the scientific field, as they not only substantially advance our understanding of the molecular mechanisms governing memory, but also newly identify a molecular pathway relevant to ARCD and cognitive dysfunction associated with TDP-43

dysfunction. miR-183/96/182 and their modes of regulation should also be investigated in other cognitive functions, especially mnemonic processes like social cognition and decision-making. In human, these functions are essential to most aspects of an active life, and are altered in many neuropsychiatric diseases.

Further, an important future development could concern investigating the role miR-183/96/182 might play in mediating the effects of environmental factors, which increase the risk of dementia, such as brain injury, traumatic stress etc. (Qureshi et al. 2010, Gardner et al. 2014). By extension, the contribution of miR-183/96/182 to the protective effects of favorable factors such as exercise and higher education on dementia risk would also be important to examine (Satizabal et al. 2016).

Finally, the identification of molecular steps through which PP1 regulates miR-183/96/182 maturation may help better understand other pathological conditions that involve aberrant microRNA processing, for example cancer, neurodegenerative disorders, cardiovascular malformations, and obesity (Li & Kowdley, 2012). Importantly in this respect, miR-183/96/182 has been associated with several forms of cancer in humans (Dambal et al. 2015). Using the regulation of miR-183/96/182 by PP1 as a prototype model to study how miRNA biogenesis is dysregulated in disease conditions may have wide-ranging implications for human health.

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Curriculum Vitae

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Medical Doctor/Neuroscientist

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Citizenship: Pakistan

Marital Status: Single

Residence permit in Switzerland: B

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EDUCATION/TRAINING

2011- 2016 Simultaneous doctoral studies at ZNZ International Program in Neuroscience, University of Zurich (UZH)/ Swiss Federal Institute of Technology (ETH), and UZH MD-PhD program, Zurich, Switzerland

2008-2010 Research fellowship/ Clinical sub-internship at Department of Neurology, Baylor College of Medicine, Houston, TX, USA

2002-2007 M.B.B.S. (Bachelors of medicine and surgery), Aga Khan University, Karachi, Pakistan (enrolled student)/ Baylor College of Medicine, Houston, TX, USA (elective student)

EMPLOYMENT

2013- Brain Research Institute, UZH/ETH, Zurich, Switzerland

2011-2012 Department of Neuropathology, University Hospital Zurich, Zurich, Switzerland

2008-2010 Department of Neurology, Baylor College of Medicine, Houston, TX, USA

HONORS

Journal Reviewer

International Journal of Biochemistry & Cell Biology, Journal of American Geriatric Society, Amyotrophic Lateral Sclerosis, European Journal of Neurology, Journal of Neurological Sciences, Neuropsychologia, Canadian

Journal of Neurological Sciences, Substance Abuse Treatment Prevention & Policy, International Journal of Tuberculosis & Lung Diseases, International Journal of Infectious Diseases, Peer J

Awards

- 2016 EMTRAN travel grant for participation in the 11th ORPHEUS congress, Cologne, Germany
- 2016 Chair of the European MD-PhD Association (EMPA)
- 2015 Chair of the EMPA Board of Representatives
- 2014 Guest Speaker Lecture at 10th International Conference of Medical Students and Junior Doctors, Lodz, Poland
- 2013 Winner of *Next Gen Voices* survey conducted by **Science** magazine
- 2012 Guest Speaker Lecture at Neurology Grand Rounds of University of Texas Health Science Center in Houston
- 2011 1st Prize in Internal Medicine category and Distinction prize in Neurology category at the 7th International Conference of Medical Students and Junior Doctors, Lodz, Poland
- 2011 Alzheimer's Drug Discovery Foundation (ADDF) Young Investigator Scholarship
- 2010 ADDF Young Investigator Scholarship
- 2010 1st Prize in Internal Medicine category at the 6th International Conference of Medical Students and Junior Doctors, Lodz, Poland
- 2010 Nomination in 'Who's Who in America' Edition 2011

SKILL-BASED EXPERTISE

Laboratory Setting

Organotypic forebrain slice cultures/ Primary neuronal cultures/ Immortalized neuronal cell lines

RNA/DNA extraction, cDNA conversion

Genotyping PCR, qPCR

Western blot, ELISA

Immunofluorescence/Immunohistochemistry

Conventional/Confocal microscopy

Mouse handling, IP injections, plantar injections, and tail-vein sampling

Mouse behavior/surgery/dissection

Clinical Setting

Clinical sub-internship in Neurology

Lumbar puncture

Arterial/Venous sampling

EEG/ECG interpretation

Structural/Functional human brain imaging

Basic Life Support (BLS)/ Advanced Cardiac Life Support (ACLS) certification

Academic/Teaching

Supervision of masters/medical students

- Vinnie Kandra (Master semester project: *Neuronal senescence induced impairment of microprocessor processing of memory-associated microRNAs*)
- Jennifer Brown (Master semester project: *Neuronal senescence induced impairment of microprocessor processing of memory-associated microRNAs*)
- Lenia Hammerstein (MD master thesis: *Metabolic dysregulation in a mouse model of early life trauma*)
- Ingrid Berg/ Julia Bollhalder/ Lukas Schmidheini/ Rebecca Muff/ Vanessa Pavolo/ Nicole Gampp/ Christina Gantner/ Jennifer Brown/ Andrea Derrer/ Aaron Brändli/ Rachel Schwagger
(UZH/ETH Block Course: *Molecular mechanism of learning & memory*)
- Andrew Michael Wilson/Emily J. McDowell/ Santosh Murthy/ Jharna Shah/ Ayesha Sajjad (Behavioral Neurology medical/research rotation)

Applied biostatistics (Lectures on *Applied Laboratory Biostatistics*)

Scientific writing (53 peer-reviewed publications)

Editorial experience (peer-review and editorials)

PUBLICATIONS**Peer-reviewed publications (PubMed Indexed Journals)**

1. Woldemichael BT*, **Jawaid A***, Kremer EA, Gaur N, Krol J, Marchais A, Mansuy IM (2016). The microRNAs cluster miR-183/96/182 contributes to long-term memory in a protein phosphatase 1-dependent manner. **Nat. Commun.** In press. *(Equal contribution) (Original study)

2. **Jawaid A.** Ingenuity results: NextGen's vision for an urban planet. **Science.** 2016; 352: 6288. (Letter to the editor)
3. **Jawaid A,** Krajewska J, Pawliczak F, Kandra V, Schulz PE. A Macro role for Microglia in Post-Stroke Depression. **J Am Geriatr Soc.** 2016 Feb;64(2):459-61. (Hypothesis article)
4. **Jawaid A,** Pawlowicz E, Schul PE. Do Acetyl cholinesterase inhibitors increase anxiety in elderly with dementia? **J Am Geriatr Soc.** 2015 Aug;63(8):1702-4. (Original study)
5. **Jawaid A,** Schulz PE. Pre-morbid type 2 diabetes mellitus as a prognostic factor in amyotrophic lateral sclerosis. **Muscle Nerve.** 2015 Jul 3. doi: 10.1002/mus.24758. (Letter to the editor)
6. **Jawaid A,** Kremer EA, Piatek A, Schulz PE. Improvement of age-related memory impairment with infusion of young plasma: a role for the peripheral amyloid sink? **J Am Geriatr Soc.** 2015;63(2):419-20. (Letter to the editor)
7. **Jawaid A,** Brown JA, Schulz PE. Diabetes mellitus in amyotrophic lateral sclerosis: Dr Jekyll or Mr Hyde? **Eur J Neurol.** 2015 Jan 18. doi: 10.1111/ene.12660. (Editorial)
8. **Jawaid A,** Gapp K, Schulz PE. Mitochondrial dysfunction and decrease in body weight of transgenic knock-in mouse model for TDP-43: the question of glucose? **J Biol Chem.** 2014;289(26):18593. (Letter to the editor)
9. Gapp K, **Jawaid A,** Sarkies P, Bohacek J, Pelczar P, Prados J, Farinelli, Miska E, Mansuy IM. Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice. **Nature Neuroscience** 2014; 17(5): 667-9. (Original Study)

10. **Jawaid A**, Paganoni S, Hauser C, Schulz PE. Trials of anti-diabetic drugs in amyotrophic lateral sclerosis: Proceed with caution? **Neurodegener Dis.** 2014; 13(4): 205-8. (Review)
11. **Jawaid A**. NextGen Voices: Results. **Science.** 2013, 339; 30-32. (Letter to the editor)
12. **Jawaid A**, Rohra DK, Zafar AM, Jawaid A. Do General Practitioners know what they are Prescribing? **J Coll Physicians Surg Pak.** 2013, 23; 375-77. (Original Study)
13. Childress JE, McDowell EJ, Dalai VV, Bogale SR, Ramamurthy C, **Jawaid A**, Kunik ME, Qureshi SU, Schulz PE. Hippocampal Volumes in Patients with Chronic Combat-Related Post-Traumatic Stress Disorder: A systematic review. **J Neuropsychiatry Clin Neurosci.** 2013; 25: 12-25. (Review)
14. Kalkonde YV, **Jawaid A**, Shirani P, Wheaton M, Pinto-Patorroyo PP, Schulz PE. Medical and Environmental Risk Factors for FTD: A Case-Control Study in a Clinical Population of Veterans. **Alzheimers Dement.** 2012; 8:204-10. (Original Study)
15. **Jawaid A**, Leske H, Neumann M. Body mass index is associated with biological CSF markers of core brain pathology in Alzheimer's disease. **Neurobiol Aging.** 2012; 33: e1-2. (Letter to the editor)
16. Shah JN, Qureshi SU, **Jawaid A**, Schulz PE. Is there evidence for late cognitive decline in chronic Schizophrenia? **Psychiatr Q.** 2012 ; 83: 127-44. (Review)
17. Neumann M, Bentmann E, Dormann D, **Jawaid A**, DeJesus-Hernandez M, Ansorge O, Roeber S, Kretschmar HA, Munoz DG, Kusaka H, Yokota O, Ang LC, Bilbao J, Rademakers R, Haass C, MacKenzie IAR. FET proteins TAF15 and EWS are selective markers that distinguish frontotemporal lobar degeneration with FUS pathology from

amyotrophic lateral sclerosis with *FUS* mutations. **Brain**. 2011; 134:2595-609. (Original Study)

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19. **Jawaid A**, Pradeep V, Shamsi A, Schulz PE. Cardiovascular risk factor associated with lower baseline cognitive performance in HIV-positive persons: A possible confounding effect of apolipoprotein genotype? **Neurology** 2011; 77: 406-7. (Letter to the editor)
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22. **Jawaid A**, Poon M, Salamone AR, Strutt AM, Pickett R, McDowell EJ, Rice LK, Simpson E, Appel SH, York MK, Schulz PE. Does apolipoprotein genotype influence the clinical expression of ALS? **Eur J Neurol**. 2011; 18:618-24. (Original Study)
23. **Jawaid A**, Murthy SB, Wilson AM, Qureshi SU, Amro MJ, Wheaton M, Simpson E, Harati Y, Strutt AM, York MK, Schulz PE. A decrease in body mass index is associated with faster progression of motor symptoms and shorter survival in ALS. **Amyotroph Lateral Scler**. 2010; 11: 542-8. (Original Study)

24. Shirani P, **Jawaid A**, Moretti P, Lahijani E, Salamone AR, Schulz PE, Edmondson EA. Familial occurrence of complex regional pain syndrome. **Can J Neurol Sci.** 2010; 37: 389-94. (Case Series)
25. Murthy SB, **Jawaid A**, Shah S, Qureshi SU, Schulz PE. Can Angiotensin-converting enzyme inhibitors prevent dementia in elderly patients with diabetes mellitus? **J Am Geriatr Soc.** 2010; 58: 613-14. (Letter to the editor)
26. Lepow L, Van Sweringen J, Strutt AM, **Jawaid A**, Macadam C, Harati Y, Schulz PE, York MK. Frontal and temporal lobe involvement on verbal fluency measures in amyotrophic lateral sclerosis. **J Clin Exp Neuropsychol.** 2010; 32: 913-22. (Original Study)
27. Murthy SB, **Jawaid A**, Qureshi SU, Bock J, Schulz PE. Marchayafava Bignami Disease in a non-alcoholic patient. **Can J Neurol Sci.** 2010; 37: 138-40. (Case Report)
28. **Jawaid A**, Rademakers R, Kass JS, Kalkonde Y, Schulz PE. Traumatic Brain Injury may increase the risk for FTD through reduced Progranulin. **Neurodegener Dis.** 2009; 6: 219-20. (Review)
29. **Jawaid A**, Salamone AR, Strutt AM, Murthy SB, Wheaton M, McDowell EJ, Simpson E, Appel SH, York MK, Schulz PE. ALS disease Onset may occur later in patients with Diabetes Mellitus. **Eur J Neurol.** 2010; 17: 733-9. (Original Study)
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33. Qureshi SU, Blanchett AR, **Jawaid A**, Schulz PE. Reversible leukoencephalopathy due to chronic unintentional exposure to Toluene. **Can J Neurol Sci.** 2009; 36: 388-9. (Case Report)
34. Zafar AM, **Jawaid A**, Ashraf H, Fatima A, Anjum R, Qureshi SU. Psychotherapy as a treatment modality for psychiatric disorders: Perceptions of general public of Karachi, Pakistan. **BMC Psychiatry.** 2009; 9: 37. (Original Study)
35. Murthy SB, **Jawaid A**, Qureshi SU, Schulz PE. The apolipoprotein 2 allele in Alzheimer's disease: suggestions for a judicious use of antiplatelet and anticoagulant medications. **J Am Geriatr Soc.** 2009 ;57: 1124-5. (Letter to the editor)
36. **Jawaid A**, Fitch O, Qureshi SU, Wilson AM, Schulz PE. Alteplase beyond 3.5 hrs in ischemic stroke: Do we know enough? **Int J Stroke.** 2009; 4: 70. (Letter to the editor)
37. Faham ZA, Takriti F, Habboub G, **Jawaid A**. Travel to Syria: expect the unexpected. **Int J Infect Dis.** 2009; 13: e326. (Letter to the editor)
38. **Jawaid A**, Zafar AM, Naveed A, Sheikh S, Waheed S, Zafar MA, Fatmi Z, Syed EU. Knowledge of Primary Pediatric Care Providers regarding Attention Deficit Hyperactivity Disorder (ADHD) and Learning Disorder (LD): A Study reported from Pakistan. **Singapore Med J.** 2008; 49: 985-93. (Original Study)

39. Rehman TU, **Jawaid A**. Disease Mongering: Role of a medical journal. **Singapore Med J**. 2008; 49: 1057-8. (Letter to the editor)
40. Murthy SB, **Jawaid A**, Schulz PE. Diabetes Mellitus and Dementia: advocating an annual cognitive screening in patients with diabetes mellitus. **J Am Geriatr Soc**. 2008; 56: 1976-7. (Letter to the editor)
41. **Jawaid A**, Rauf MA, Usman U, Khealani BA. Post-infarct cerebellar cognitive affective syndrome: a case report. **J Pak Med Assoc**. 2008; 58: 415-7. (Case Report)
42. **Jawaid A**, Zafar AM, Rehman TU, Nazir MR, Gafoor ZA, Afzal O, Khan JA. Knowledge, attitudes and practice of university students regarding waterpipe smoking in Pakistan. **Int J Tuberc Lung Dis**. 2008; 12: 1077-84. (Original Study)
43. **Jawaid A**, Schmolck H, Schulz PE. Hypersociability in Williams Syndrome (WS): A role for the Amygdala? **Cognit Neuropsychiatry**. 2008 Jul;13(4):338-42. [PMID: 18622788]. (Comment)
44. **Jawaid A**, Zafar AM, Mahmood SF. Impact of Afghan refugees on the infectious disease profile of Pakistan: beyond economy. **Int J Infect Dis**. 2008; 12: e131-32. (Letter to the editor)
45. **Jawaid A**. Cervical Cancer Vaccine in Pakistan: Let's start thinking. **Int J Infect Dis**. 2008; 12: 217. (Letter to the editor)
46. **Jawaid A**, Khuwaja AK. Treatment and Vaccination for Hepatitis C: present and future. **J Ayub Med Coll Abbottabad**. 2008; 20: 129-33. (Review)
47. **Jawaid A**. Literacy and Misunderstanding Prescription Drug Labels. **Annals Intern Med**. 2007, 147: 280. (Letter to the editor)

48. **Jawaid A**, Rehman TU. Physician-Pharmaceutical Interaction: Training the doctors of tomorrow. **J Pak Med Assoc.** 2007, 57:380-1. (Letter to the editor)

49. **Jawaid A**, Rehman TU. Paediatric mental health in Pakistan: A neglected avenue. **J Pak Med Assoc.** 2007;57: 50-1. (Letter to the editor)

50. **Jawaid A**, Aftab O. Water-Pipe smoking: A threat never realized. **J Pak Med Assoc.** 2006;56: 341-2. (Letter to the editor)

51. **Jawaid A**, ur Rehman T, Rohra DK. Regulation of human coronary vascular tone: further evidence must be sought before ruling out the direct role of ATP-sensitive potassium channels in regulation of coronary vasculature. **Circ Res.** 2006;98: e73. (Letter to the editor)

52. Rohra DK, **Jawaid A**, Tauseef-ur-Rehman, Zaidi AH. Reliability of rodent animal models in biomedical research. **J Coll Physicians Surg Pak.** 2005;15: 809-12. (Review)

53. Aftab O, **Jawaid A**. Rabies control in Pakistan--the wise strategy. **J Coll Physicians Surg Pak.** 2005;15:748. (Letter to the editor)

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Average Citation per publication: 14.83

H-Index: 13

First Author Publications: 32

Book chapter

1. Riby DM, Bruce V, **Jawaid A**. Everyone's friend: The case of Williams syndrome. In: Oakley B, Knafo A, Jhonson D, Madhavan G, Wilson DS editors. **Pathological Altruism. 1st ed. Oxford Publishers; 2012**

RESEARCH SUPPORT (Grants written or co-written)

- 2014-2016: Swiss National Science Foundation for ***Study of the role of protein phosphatase 1 in microRNAs regulation during memory formation in mice***. Main Personnel. PI: Prof. Isabelle Mansuy
- 2008-2010: DeGeorge Foundation grant for ***Dementia Research***. Co-PI. PI: Dr. Paul Schulz
- 2008-2009: Kozmetsky Foundation grant for ***Frontotemporal Dementia in Amyotrophic Lateral Sclerosis Patients***. Co-PI. PI: Dr. Paul Schulz

Acknowledgements

My first and foremost thanks goes to Isabelle, not only for allowing me to complete this exciting work in her lab, but also for being an exemplary supervisor. Working with her carries a unique balance of freedom and guidance. As one of her 'kids', as she amusingly calls her students sometimes, I was encouraged to hypothesize, prompted to be organized, reminded to be rigorous, and on occasions compelled to repeat the experiments, just to be sure. This drill, though challenging, has ensured a fidelity and reproducibility of all the data I produced in her lab. She is also quick to identify the strengths and aspirations of her students. Knowing my passion for teaching, and my inclination towards mechanistic and translational research, she provided me ample opportunities to teach, and unconditionally supported me with adding translational experimental aims to my thesis proposal. I am also indebted to her for being extremely patient and sympathetic when I fractured my elbow in the second year of my PhD.

I am also extremely grateful to Sebastian and Constance for their invaluable comments and insight during my committee meetings. It was nothing less than a blessing to have an accomplished physician-scientist Sebastian and a remarkable cell biologist Constance in my committee, as my thesis had both translational and mechanistic dimensions. Besides giving critical scientific input, they also helped me a lot with maintaining my motivation throughout this challenging thesis.

I also highly regard the contribution of Bisrat, with whom I largely shared this project. Indeed, the initial identification of miR-183/96/182 as memory-promoting miRNAs was Bisrat's finding, and served a basis for the rest of the experiments.

I also cannot ignore the energy, enthusiasm, ideas, and zeal my students brought to this project. They were very diverse in personalities, but coalesced in their commitment and will to learn. Both Jenny and Vinnie could be counted on to fulfill their responsibilities with precision, accuracy, and rigor. Jenny, in

particular, also contributed significantly to the intellectual basis of Chapter 3 of this thesis. I was also fortunate enough to collaborate with Niha in the last year of my thesis, who performed countless Western blots, and qPCRs for me. I would not have managed this project without her contribution. I must also mention a couple of my block course students, who impressed me enormously with the level of knowledge and skill they possessed for their age. Ingrid and Julia, I am placing my bets on you becoming professors one day!

I always jokingly admit that I literally 'sneaked' my way into another vastly intriguing project at the Mansuy lab. This project, which newly identified sperm RNA as vectors of trans-generational effects of early life trauma in mice, was published in *Nature Neuroscience*, and allowed me the delightful collaboration and friendship of Kathi. I also had the pleasure of supervising a remarkably talented medical student Lenia on a transgenerational inheritance project. She is another one destined for laurels!

The first block-course I supervised was also the start of my friendship and collaboration with Eloïse. Together, we have developed novel luciferase assays, written scientific comment articles, trouble-shooted qPCRs, disinfected cell culture rooms, supervised four block courses, freaked out about our HIFO day talks, invented games for the lab retreat, made movies for Kathi's and Nadia's defense, made fun of Lukas, discussed my one thousand and one love interests, cooked Pakistani food and appreciated (by force, in my case) French goat cheese, roamed the streets of Amsterdam, and.....It's a never ending list. All I can say is, Thank you Elo, because of you, it worked more than it didn't 😊 Here, I would also mention the 3rd member of the brotherhood, Lukas, who balanced our craziness with pragmatism 😊, drove us on his car around whenever (politely) asked, made me sweat in ping-pong, and helped me countless times with English-German translations.

Life in and around HIFO would not be the same if it wasn't for; Martin, my weekend work-buddy, and Silvia, our graceful and uber-organised lab manager. Outside the Mansuy lab, I cherish the moments spent with: Anne, a

total renaissance woman with an intellect three million light years out of my reach, Alex, the happiest thing about HIFO happy hours, and Nadia, my office/gym/gossip/i-give-you-my-fruit-because-you-look-so-sad buddy.

A thoroughly enriching part of my MD-PhD life was my involvement with the European MD-PhD association (EMPA), which gave me, besides thrilling MD-PhD conferences, fascinating bunch of friends, Fritz, Myrthe, Ruben, Mathilde, and Vlad.

A pivotal figure in my academic career is my mentor from Baylor, Dr. Paul Schulz. He took me under his wing when I was a clueless medical student, who knew nothing about research, other than that he wanted to do it. My scientific acumen, methods, and ethics are all attributed to his guidance, support, and mentorship.

Perhaps, the biggest challenge I have faced in my entire life is to match up to the legacy of being a Jawaid. Abu (my father) had degrees in English literature, public administration, and law, and an unmatched intellect and grace. It is not a surprise that my siblings turned out to be a creative painter, an impressive literalist, a brilliant surgeon, and an award-winning engineer. The only way I could have matched up to them was doing a double doctorate, and if I succeed in that today...I would owe it to the inspiration and support I got from you Samra, Sidra, Arfat, and Shoaib.

There is a reason why I am mentioning Ami (my mother) as the last acknowledgement, for a purely symbolic purpose. This thesis started with my dedication to her, and it is just fitting that it ends with her mention. Whatever little good I have done in this world, as a scientist, and a person, I owe it to Ami. Her blissful presence, prayers, tea refills, delicious meals, astounding advices, Punjabi jokes, fashion recommendations (yes, it's true ☺), and many many more...have made me who I am today. I can't thank you enough Ami!

If this thesis were a fairy tale, I would be writing 'and they lived happily ever after' right now. I don't know, and perhaps also don't care, about the 'ever

after' part: I just know that owing to all those who I mention here, or might have inadvertently missed, I 'lived' this MD-PhD, a life which has been stimulating, enriching, challenging, and satisfying, and I promise to give my utmost to ensure that this life you all gave me will make a difference to other lives in future, in the best possible way.